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ENTRY OF PHOSPHATE INTO YEAST CELL[1]

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Baker's yeast, *Saccharomyces cerevisiae*, has often been selected for study of active, or metabolically associated, salt accumulation. Particular attention has been devoted to the esterification of phosphate in this process and to the nature of coupling with a source of energy. The accumulation of phosphate has been considered as coupled to: A, the general reactions of glycolysis (10); B, the action of glyceraldehyde–3–phosphate dehydrogenase (6,16); C, a redox pump (4,5) and D, oxidations in the respiratory chain under aerobic conditions (14).

With these concepts in mind, the manner of phosphate entry into yeast was examined by the methods of quantitative enzyme kinetics applied by C. E. Hagen and his associates (7) to analysis of phosphate entry into barley roots. The initial objective was to compare phosphate accumulation by Baker's yeast and barley roots, particularly with respect to aerobic and anaerobic conditions, in the hope of more clearly understanding the aerobic requirement of barley roots. Results obtained indicate that phosphate entry into yeast is coupled with three distinct reactions of oxidative phosphorylation, one of which is aerobic and the other two anaerobic.

METHODS

A *Saccharomyces cerevisiae* Meyer ex Hansen was isolated from Baker's yeast (Standard Brands, Inc.) and grown aseptically. The original yeast contained cell fragments and a bacterial contaminant which confounded all experiments until it was eliminated. Cultures were grown with aeration on glucose-bactopeptone medium containing beef extract and 0.1 M in NaCl for 24 hours at 30° C. Cells were removed by centrifuging, followed by washing and resuspension in 0.15 M NaCl. Concentrations of cells were measured turbidmetrically and adjusted to contain 10 mg dry weight/5 ml.

The cells (5 ml) were pretreated in 30 ml of a requisite solution for 60 minutes to establish steady-state conditions with respect to orthophosphate uptake (fig 1). Steady-state persisted for 60 minutes, after which the rate decreased slightly. Constant uptake is reached in shorter times at lower glucose concentrations. Uptake of P^{32} was followed for periods of 30 to 300 seconds and was terminated by centrifugation. Cells were washed once with 0.15 M NaCl. Prolonged washing did not further reduce the amount of phosphate associated with the yeast cells. The phosphate remaining with the cells is described as that accumulated. Radioactivity was measured after drying under infra-red lamps. The amount of P^{32} associated with the cells was converted to moles per mg of cells from the known specific activity which was constant during the absorption period. Oxygen consumption was measured polarographically. Anaerobic conditions were established by bubbling oxygen-free nitrogen through a cell suspension in a 35 ml volume of small surface area.

KINETIC INTERPRETATION: Active accumulation of ions by cells is considered to arise from initial combination with a specific binding compound (carrier) at an external surface, followed by breakdown delivering the ion inside the cell. The reaction

$$X \text{ (carrier)} + R_o^- \text{ or } R_o^+ \text{ (external ions)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}}$$

$$XR^{\pm} \text{ (carrier compound)} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} X' + R_i^- \text{ or } R_i^+ \text{ (inside)}$$

[1] Received August 15, 1960.

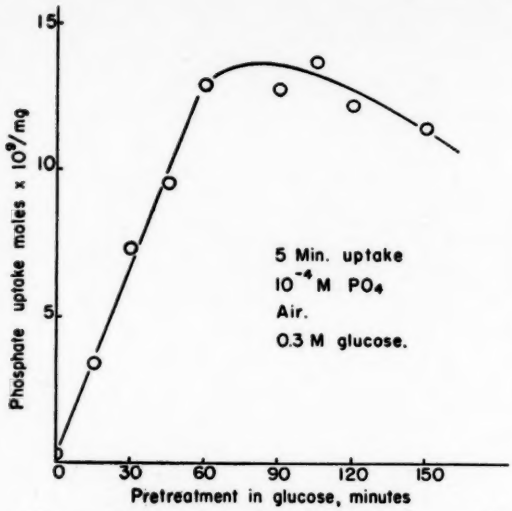


FIG. 1. Dependence of phosphate uptake by yeast in 5 minute periods on the time of incubation on 0.3 M glucose under aerobic conditions at pH 4.0.

Under steady-state conditions the back reaction involving k_4 is negligible. The amount, m, of an ion accumulated by the cell in time, t, is

$$m = k_3 [XR]t + [XR]$$

where [XR] is the concentration of the carrier compound. Under steady-state conditions the uptake follows the Michaelis-Menten equation,

$$m = -\frac{K_m m}{[C_o]} + m_{max}$$

where $[C_o]$ is the constant external ion concentration of an infinite reservoir. The maximum uptake, m_{max}, is obtained by extrapolation of $[C_o]$ to infinite concentrations.

A linear plot of m against $\frac{m}{[C_o]}$ indicates a single reaction with the Michaelis-Menten constant $K_m = \frac{k_2 + k_3}{k_1}$ being the negative slope and m_{max} the intercept on the axis of ordinates. A curvilinear relationship results when two or more first-order reactions occur simultaneously but independently (8). These curves can be resolved into linear components with a reasonably critical attitude toward precision and extent of measurements and possible departure from first-order kinetics.

The uptake of phosphate, m, is measured as a function of phosphate concentration under various conditions of the environment (pH & temperature, aerobiosis & anaerobiosis), and with various substrates, inhibitors, and competing materials. Analysis of the results indicates the number of rate-limiting processes and the corresponding values of K_m and m_{max}. Values of k_3 and [XR] can be found from the phosphate uptake as a function of time.

RESULTS

I. INITIAL CONDITIONS: Yeast showing the maximum rate of phosphate uptake (fig 1) and respiration can readily be depleted of endogenous substrates by suspending in 0.15 M NaCl for 60 minutes. Respiration of these cells responds immediately to additions of glucose, glutamate, and other substrates. It is constant from zero time and responds without lag to the various inhibitors.

Rates of oxygen consumption referred to the rate with 0.3 M glucose as 100 are shown in table I. The concentrations of the several compounds include those used during measurement of phosphate uptake.

II. PHOSPHATE UPTAKE UNDER AEROBIC & ANAEROBIC CONDITIONS: The ratio of the slopes of the time curves to their intercepts at zero time, $k_3 [XR]/[XR]$ are constant with varying phosphate concentrations and constant substrate. This indicates the absence of non-specific adsorption in the measurement of [XR]. The amount of phosphate held at zero time, [XR], is increased with anaerobiosis (fig 2 & 3). Irrespective of the phosphate concentration, the rates of phosphate uptake, $\frac{dm}{dt} = k_3 [XR]$, after zero time are the same under aerobic and anaerobic conditions as shown by the parallel displacement of the time curves.

If glucose is withheld the rate of phosphate uptake drops to zero (figs 2 & 3) and the value of [XR] is

TABLE I

OXYGEN CONSUMPTION IN AIR BY YEAST WITH VARIOUS SUBSTRATES AT pH 4.0 & 30°

SUBSTRATE	MOLAR CONC	OXYGEN CONSUMPTION 2.3×10^{-8} moles O_2/min/mg
Glucose 0.3 M		
Without inhibitor		100
5,5-diethyl barbituric acid	2×10^{-3}	85
Sodium azide	10^{-3}	0.01
Dinitrophenol	10^{-4}	70
Without glucose		
β-hydroxy butyrate	0.1	100
Ethanol	0.9	100
Sodium chloride	0.15	0.01
Glutamic acid	10^{-3}	80
Succinic acid	2×10^{-2}	0.02
p-phenylene diamine (reduced)	1×10^{-2}	70

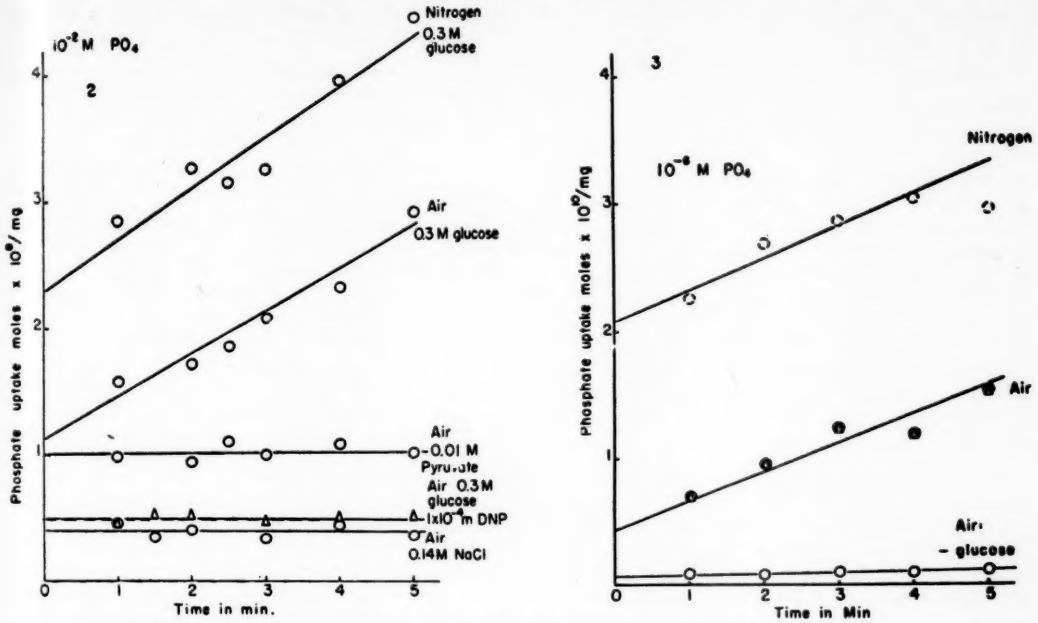


FIG. 2. Phosphate uptake by yeast from 10^{-2} M phosphate at pH 4 as a function of time.
FIG. 3. Phosphate uptake by yeast from 10^{-6} M phosphate, pH 4, as a function of time on 0.3 M glucose. Aerobic and anaerobic.

reduced. A similar result is obtained in the presence of glucose and 1×10^{-4} 2,4-dinitrophenol (DNP) (figs 2 & 10). The rate is also reduced to zero with 0.01 M pyruvate as the substrate (fig 2), however, without changing [XR].

Plots of uptake, m, against $m/\Sigma[PO_4]$ are similar under aerobic and anaerobic conditions (figs 4 & 5). The curves can each be resolved into two linear portions, having K_m values of 10^{-3} and 10^{-5}, respectively. Values of m_{max} for both systems decrease if the

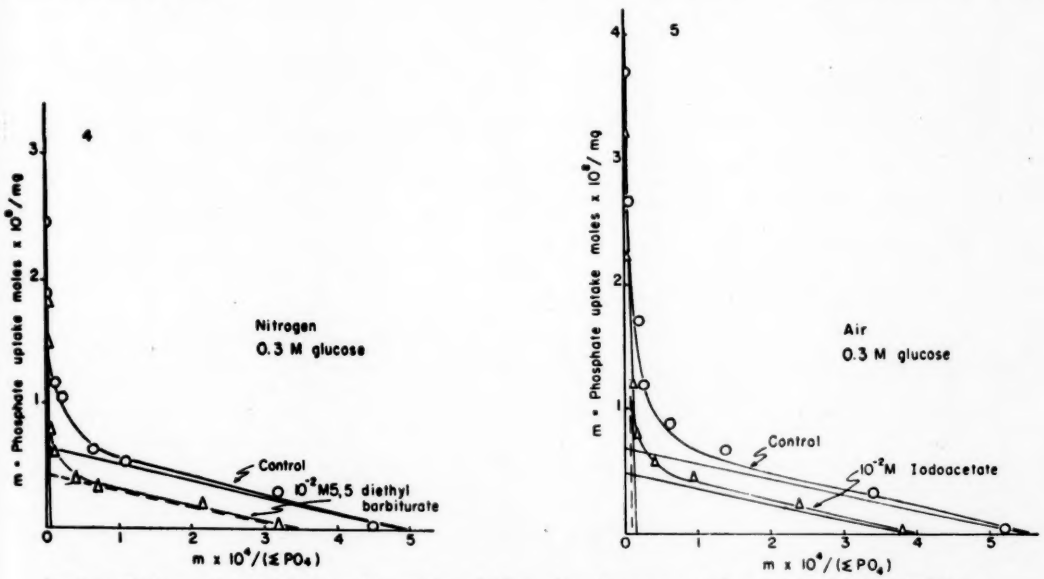


FIG. 4. Hofstee plot of the anaerobic uptake of phos phate by yeast as influenced by 5,5-diethylbarbituate.
FIG. 5. Hofstee plot of the aerobic uptake of phosph ate by yeast as influenced by iodoacetate.

glucose concentration is reduced below 0.3 M without change in values of K_m. Intermediate concentrations of glucose (10^{-3}–10^{-2} M) primarily decrease the value of [XR] while at 10^{-4} M both [XR] and k_3 decrease as shown by comparisons of the time curves. In the absence of glucose the constant, k_3, is not measurable. Thus the K_m value can be determined and expressed solely as a function of k_1 and k_2, i.e., $K_m = k_2/k_1$. The K_m values are constant at all glucose concentrations with the implication that even at the highest turnover rate, the k_3 is insignificant relative to k_2 and K_m is a dissociation constant.

A third reaction with a value of K_m of 10^{-6} is observed only with low glucose concentrations under aerobic conditions (fig 6). When the glucose concentration is increased this reaction is inseparable from the intermediate reaction having $K_m = 10^{-5}$. The increase in glucose increases m_{max} far more for the latter than for the former reaction.

III. Action of Respiratory Inhibitors on Reaction With $K_m = 10^{-6}$: In the absence of glucose and with 2×10^{-6} M PO_4, the value of k_3 is negligible (fig 7). Addition of 0.1 M *p*-phenylenediamine increases the intercept [XR] by about fourfold. A glucose concentration of 10^{-4} M produces an observable value of k_3. The slope/intercept ratio is not changed by addition of *p*-phenylenediamine. This indicates an increase of [XR] with a constant value of k_3.

This reaction is inhibited by 5,5–diethyl barbituric acid (barbital) primarily by decrease of [XR] and by azide with resulting decrease in [XR] and k_3.

IV. Action of Glycolytic Inhibitors on Reaction With $K_m = 10^{-5}$: Iodoacetate, 5,5–diethyl barbituric acid and Na_2SO_3 are equally effective as inhibitors under aerobic and anaerobic conditions. Each effects a decrease in [XR] and, at high concentrations, causes k_3 values to approach zero (figs 4, 5, & 8). Inhibition of phosphate uptake is of the uncompetitive type; that is, m_{max} is changed by the inhibitor while K_m remains constant.

The action of Na_2SO_3 differs from that of the other two inhibitors. In the presence of Na_2SO_3 the curve on the plot of m against $m/\Sigma[PO_4]$ is resolvable into three components. This might be a result of more effective inhibition of the reaction with $K_m = 10^{-5}$, allowing the reaction with $K_m = 10^{-6}$ to be displayed. However, iodoacetate and the barbituate might decrease [XR] for both reactions so that they are inseparable.

High concentrations of *p*-phenylenediamine are without effect on phosphate uptake in the presence of 0.3 M glucose.

V. Action of Inhibitors on Reactions With $K_m = 10^{-3}$: This reaction has $m_{max} = 27 \times 10^{-8}$, $k_3 = 3.2 \times 10^{-3}$, and $[XR] = 13.8 \times 10^{-8}$ moles/mg of yeast. The k_3 for this reaction is smaller than for the other two. The glycolytic inhibitors at the concentrations indicated in table I have little effect (figs 4 & 5) but moderate changes would be difficult to note.

A concentration of 10^{-4} M DNP decreases [XR] and blocks k_3 (fig 2). Thus, the reaction must be connected with a metabolic step rather than with diffusion or other mode of passive entry.

VI. Action of ATP, DNP, Succinate, & β–Hydroxybutyrate: All reactions are inhibited in a competitive manner by ATP and are blocked by DNP. Succinate supports some respiration (table I) but is without effect on phosphate uptake (fig 10). Beta hydroxybutyrate, on the other hand, markedly inhibits phosphate uptake in the presence of 0.3 M glucose by decreasing [XR] without changing the slope/intercept ratio of the time curve (fig 10). Respiration is sustained to the same level by beta-hydroxybutyrate and 0.3 M glucose (table I).

VII. Effect of Change in pH & Presence of KCl: The optimum pH for phosphate uptake depends upon the concentration of the external solution. As phosphate decreases from 10^{-2} to 10^{-6} M the optimum shifts from pH 5.0 to 6.5 in both anaerobic and aerobic systems. The utilization of glucose from 0.3 M solution is also optimal at pH 6.5.

Potassium chloride is without effect on the pH dependence of phosphate uptake. At pH 4.0 in the presence of 0.3 M glucose and 10^{-6} M PO_4 a concentration of 0.1 M KCl enhances phosphate uptake by increase of [XR] corresponding to a constant ratio of slope/intercept of phosphate uptake (fig 9).

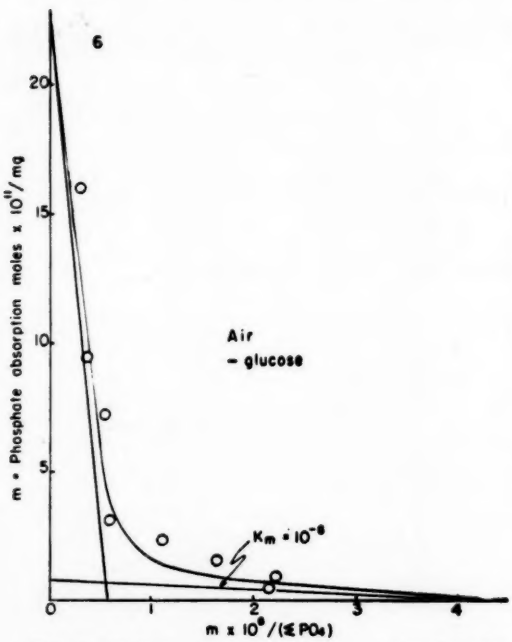


Fig. 6. Hofstee plot of the uptake of phosphate by yeast in the absence of a substrate. Aerobic.

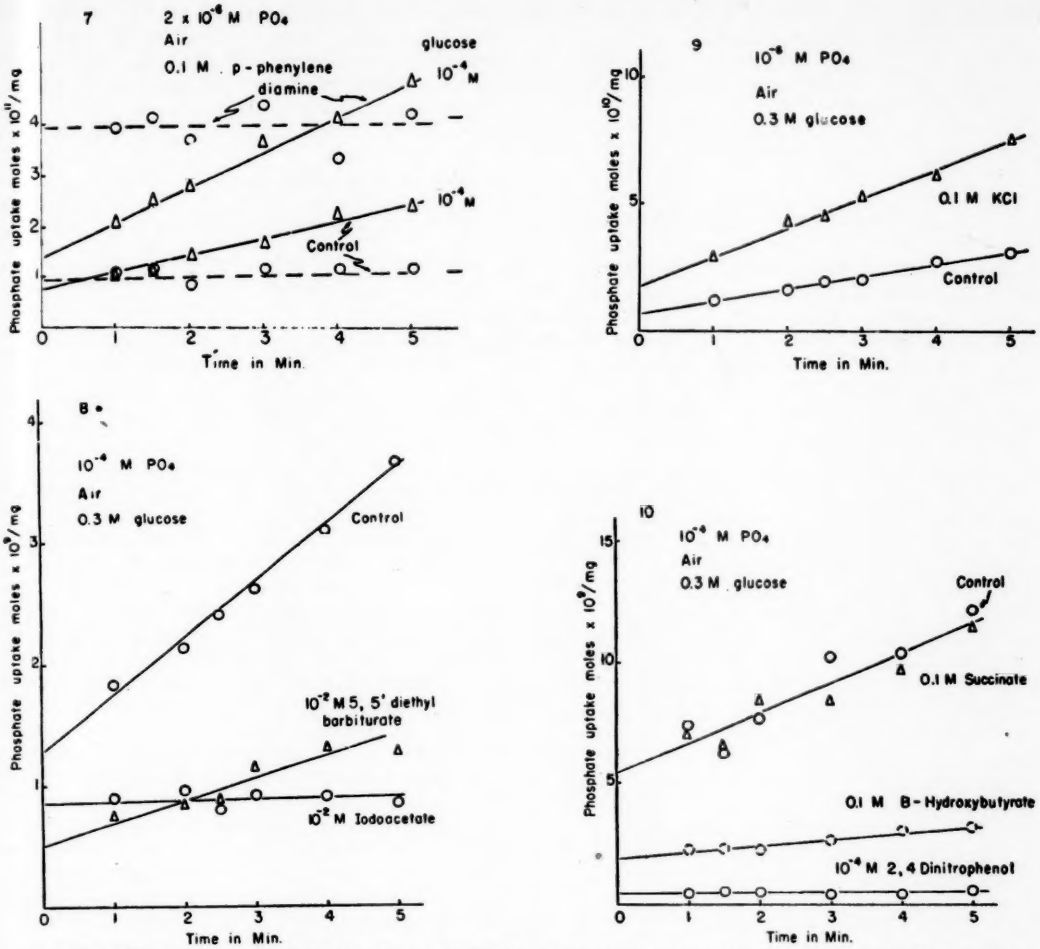


FIG. 7. The effect of *p*-phenylenediamine on the uptake of phosphate by yeast in the presence and absence of glucose. Aerobic.

FIG. 8. The effect of barbital and of iodoacetate on the uptake of phosphate by yeast. Aerobic.

FIG. 9. The effect of KCl on the uptake of phosphate by yeast. Aerobic.

FIG. 10. The effects of succinate, β-hydroxybutyrate, and 2,4-dinitrophenol on the uptake of phosphate in the presence of 0.3 M glucose. Aerobic.

DISCUSSION

Absorption of orthophosphate by Baker's yeast appears to be coupled with the oxidative formation of ATP chiefly by the action of glyceraldehyde-3-phosphate-dehydrogenase under both aerobic and anaerobic conditions, as was earlier concluded in part by Goodman and Rothstein (6). The reaction with $K_m = 10^{-6}$, however, has characteristics indicating coupling with the oxidative formation of ATP associated with cytochrome b of the respiratory chain. The nature of the reaction with $K_m = 10^{-3}$ and of the varied pattern of phosphate uptake by yeast and barley are of particular interest.

The general pattern for the coupling mechanism in oxidative phosphorylation associated with the respiratory chain has been formalized by Lehninger (13), Slater (19), Martius (15), and Lardy (12), [also note Chance & Williams (2)] as follows:

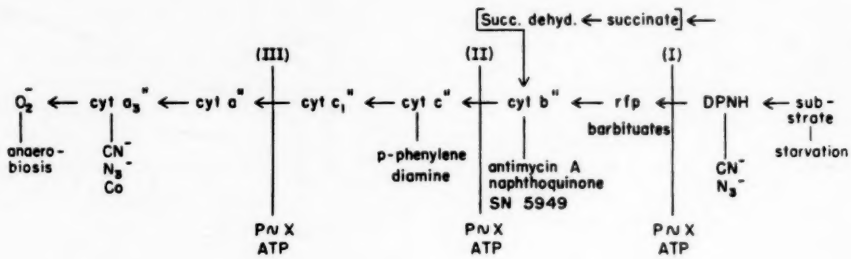
$$\text{Carrier} + X \xrightarrow[\text{transfer}]{\text{electron}} \text{carrier} \sim X \qquad [1]$$

$$\text{Carrier} \sim X + P_i \rightleftharpoons \text{carrier} + P \sim X \qquad [2]$$

$$P \sim X + ADP \rightleftharpoons X + ATP \qquad [3]$$

$$ATP + \text{glucose} \rightleftharpoons \text{glucose-6-phosphate} + ADP \qquad [4]$$

Three positions of oxidative phosphorylations in the series of oxidations and reductions of the respiratory chain as established by Slater (19) and others (1) are indicated by (I), (II), and (III) in the following scheme:



Positions of action of various inhibitors are indicated below the reaction sequence. Because succinate does not support much oxygen consumption in Baker's yeast (table I fig 10), under the conditions used its action is not further considered.

Several features of interaction of compounds influencing uptake of phosphate by intact cells by action as inhibitors or otherwise must be held in mind. Several respiration inhibitors were found by Chance (1) to have the same action on intact cells and mitochondria of ascites cells. The inhibitor studies are of short duration, which minimizes overall metabolic effects and permits use of compounds which eventually might be toxic. The conditions of use require the inhibitor to be effective as quickly as is phosphate uptake, which is essentially from time zero.

The inhibitor and phosphate both act as if their concentrations are the same or are linearly connected with those of the external solution. This implies that diffusion processes and pathways requiring appreciable time for establishment of steady-state conditions are not limiting. The uptake system can be expected to have a spatial separation of sequential reactions differing from that of a homogenous system such that an inhibitor might be more specific for a particular reaction. This is particularly true for respiration which might, in part, be closely associated with uptake but also arising, in part, from deep in the cell where it is not subject to immediate inhibitor action.

Uptake of phosphate with low concentrations of glucose and phosphate gives a linear component on a Hofstee plot (8) with $K_m = 10^{-6}$ (fig 6). This component reaction is strictly aerobic. Paraphenylenediamine increases the site concentration [XR] without changing the k_3 of [XR]. The maximum site increase is in the absence of glucose (fig 7). The changes are consistent with enhanced reduction of cytochrome b in the presence of the inhibitor, and are consistent with the in vitro observation that the site of action of *p*-phenylenediamine is between cytochromes c and b (11). If this is the case one should be able to follow the oxidation and/or reduction of phenylenediamine in the presence of the yeast cells. This has been observed, using the oxygen electrode. In the absence of exogenous substrate, reduced phenylenediamine supports respiration (table I). On the other hand, yeast will reduce oxidized phenylenediamine at a rate (2×10^{-8} moles/min/mg) equivalent to oxygen uptake by cells in a glucose medium. On the basis of the potential, one would predict that phenylenediamine could function in the proposed region of the respiratory chain. At the present time spectral changes of cytochromes c and b induced by *p*-phenylenediamine were not observed due to its absorption in the region of 560 mμ.

Barbital inhibits the uptake of phosphate at low concentrations of phosphate and glucose by decreasing [XR]. Pyruvate and ethanol, in the absence of glucose, under aerobic conditions increase the site concentration without leading to a measurable rate of turnover of [XR] (fig 2). Failure of turnover possibly arises from depletion of adenosine diphosphate (ADP) in reaction [3] because of the absence of reaction [4]. The several actions are consistent with phosphate uptake associated with change in oxidation of cytochrome b. Uptake of phosphate associated with change in oxidation of DPNH coupled to the respiratory chain could not be examined because of predominant coupling of DPN reduction with an anaerobic reaction ($K_m = 10^{-5}$).

The component of phosphate uptake with $K_m = 10^{-5}$ is present under both aerobic and anaerobic conditions (figs 4 & 5). It is inhibited by iodoacetate (figs 5 & 8) and sulfite and is unaffected by acetate. Iodoacetate probably is effective by reaction with the —SH group of glyceraldehyde-3-phosphate dehydrogenase while sulfite combines with the aldehyde and with acetaldehyde. It is of particular interest that while iodoacetate is inhibitory in both anaerobic and aerobic systems, acetate is effective only in the latter. These results are in agreement with Prankerd and Altman (16) and with the conclusions of Goodman and Rothstein (6) that the phosphate uptake is associated with the action of glyceraldehyde-3-phosphate dehydrogenase.

The anaerobic uptake of phosphate is inhibited by 2,4-dinitrophenol at 1×10^{-4} M and by 5,5,diethyl-

barbituate (figs 4 & 8). The barbituate changes the site concentration and m_{max} without changing K_m (fig 4) or k_3 (fig 8). Both are somewhat inhibitory to respiration. Dinitrophenol when used aerobically with separated mitochondria (2) uncouples respiration and oxidative phosphorylation, while with root disks it uncouples respiration and salt uptake (17). It is widely considered to be without effect on substrate level phosphorylations where most attention has been devoted to α-ketogluterate decarboxylation and subsequent oxidation to succinate (3). Barbituates are used as inhibitors of flavin mononucleotide (FMN) action in the respiratory chain. Neither, accordingly, would be anticipated to affect anaerobic formation of adenosine triphosphate (ATP) and, in fact. were used with this initial expectation.

Dinitrophenol when used in studies of phosphate uptake by barley roots (9) decreased uptake competitively with phosphate. This possibility has not been suggested in the extensive work with separated mitochondria.

The reason for a possible oversight might be that in the work with mitochondria the phosphate level is usually greater than 10^{-3} M and that of DNP is low (1×10^{-5} M or less) so that inhibition would be masked. The K_I value with intact barley roots found by Hopkins is 1.14×10^{-6} moles. DNP then, irrespective of uncoupling action, appears to be competitive with phosphate and this would seem to be its action on the anaerobic components of phosphate uptake with $K_m = 10^{-5}$ and 10^{-3}.

The action of the barbituates in inhibiting anaerobic phosphate uptake by yeast is not understood in terms of reactions immediately associated with action of glyceraldehyde–3–phosphate dehydrogenase. It might be that in the uptake action an FMN protein is connected with the reduction of diphosphopyridine nucleotide (DPN).

The values of k_3 for the aerobic and anaerobic reactions with $K_m = 10^{-5}$ are the same. This indicates that only one reaction is involved which, accordingly, must be in the glycolytic pathway. The reaction with $K_m = 10^{-6}$ also has this same value of k_3 under aerobic conditions. The indication is that this is a result of the sequential nature of the reactions running from glucose to oxygen.

Two steps are recognized for ATP formation resulting from dehydrogenation of phosphoglyceraldehyde. The first is connected with reduction of DPN mediated by glyceraldehyde–3–phosphate dehydrogenase, and the second by reaction with ADP and 1,3 phosphoglyceric acid and release of ATP mediated by 3, phosphoglyceric 1–kinase. The inhibitory action of ATP on the reaction with $K_m = 10^{-3}$ indicates that the last step is involved. A chance of confusion exists if ATP dilutes the added P^{32} either by hydrolysis or exchange, but with the high phosphate concentrations involved the dilution even with complete hydrolysis would have been negligible.

The component of phosphate uptake with $K_m = 10^{-3}$ is markedly effective only at phosphate concentrations $> 10^{-4}$ M in the presence of 0.3 M glucose. It would have appreciably contributed to the phosphate uptake by yeast observed by Goodman and Rothstein (6) and would have been greatly predominant in some of their experiments. Neither iodoacetate nor barbital apparently inhibits the reaction at concentrations of 10^{-1} M, although precision is so low that a one and a half-fold change would be uncertain (figs 4 & 5). The value of k_3 for the reaction is lower than that for the other two, which indicates that the rate-limiting reactions must be different.

At high phosphate concentrations the amount of ADP available for phosphorylation is relatively low. Furthermore, glucose is optimal for respiration, which suggests that hexokinase must be limiting for entrance of glucose into the glycolytic pathway. Under these conditions the hexokinase is also limiting for phosphate uptake. Inhibitors would not be adequately effective to put the controlling rate in earlier reactions by limiting either glycolysis or electron flow in the respiratory chain.

The general concepts of phosphate uptake by Baker's yeast can now be formulated in accord with the preceding analysis and compared with phosphate uptake by barley roots as previously formulated by Hagen, Leggett, and Jackson (7). Only a small fraction of the oxidative phosphorylation associated with the respiratory chain is coupled to phosphate uptake because the respiratory system is present only to a small extent at the surface of the limiting membrane. None of the experiments reported here bears on the extent to which respiration is coupled with ATP formation by turnover of endogenous phosphate, but the results of Chance and Williams (2) indicate that the P/O ratio might approach 3.0. In barley roots, on the other hand, the mitochondria are apparently embedded in the limiting membrane, possibly by the vacuolating of the cell. In yeast the oxidative phosphorylation associated with glyceraldehyde–3–phosphate dehydrogenase is present at the membrane surface, as also is the hexokinase reaction which utilizes ATP and introduces glucose into the cell. Glucose, in the absence of phosphate, can still enter through use of endogenous ATP. These deductions are in accord with those of Rothstein (18) and others. Barley roots, in contrast, while having a glycolytic pathway for utilization of glucose (20), apparently do not have the oxidative phosphorylation reaction coupled to glyceraldehyde–3–phosphate dehydrogenase at the cell surface to any considerable extent. In short, the barley root has the components of the respiratory chain at the surface of the limiting membrane while the yeast has the oxidative phosphorylation step of glycolysis.

Summary

Phosphate uptake by Baker's yeast, *Saccharomyces cerevisiae*, was measured in pure cultures under aerobic and anaerobic conditions. Effects of various substrates and respiratory chain and glycolytic inhibitors were examined.

Analysis of the results by means of formulation of first-order kinetics indicate three components of phosphate uptake. The minor one of the three is associated with oxidative phosphorylation coupled to cytochrome b of the respiratory chain. It is effective only under aerobic conditions. The other two components which constitute the main course of phosphate uptake at concentrations above 10^{-5} molar are effective under both aerobic and anaerobic conditions. Action of inhibitors indicate that they are both associated with oxidative phosphorylation coupled to the action of glyceraldehyde-3-phosphate dehydrogenase. The rate of one of the two components is interpreted as being limited by the phosphorylation of adenosine diphosphate. The rate of the other component, which is predominant at high phosphate concentrations ($> 10^{-3}$ M) and optimal glucose concentrations (0.3 M), appears to be limited by the action of hexokinase.

Acknowledgment

This investigation was started in association with the late C. E. Hagen. Demitros A. Soulides isolated the pure cultures of *Saccharomyces cerevisiae* Meyer ex Hansen, and advised on sterile technique. Advice in experimental design and the critical interest of P. C. Jackson and S. B. Hendricks are gratefully acknowledged.

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RESPONSES OF SEEDS OF PINUS VIRGINIANA TO LIGHT [1]

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The responses to light of lettuce and Lepidium seeds depend on the reversible red, far-red photoreaction (3,5). These light-sensitive seeds are rather small in comparison with seeds of several species of southern pines reported by Nelson (4) to be stimulated to germinate by light. The current experiments were conducted to determine whether seeds of *Pinus virginiana* respond to the reversible red, far-red photoreaction in the same way as lettuce and Lepidium seeds, and how the responses are modified by temperature and condition of imbibition.

MATERIAL & GENERAL METHODS

Four samples of seeds of *Pinus virginiana* Mill. were obtained from the Beltsville Experimental Forest. Seeds of sample LP-13, collected in the fall of 1955, were held at 1° C until March 1956, when they were received at Plant Industry Station and placed in a sealed can at −18°. Seeds from this sample were used in experiments during April to August 1956. Seeds of sample LP-62, collected in October 1957, were kept continuously at 1°, and those of sample LP-63, collected in October 1958, were kept at room temperature. Seeds from the last two samples were used in experiments during November 1958 to January 1959. Seeds of sample LP-64, collected in October 1958, were placed in a sealed can at −18° in November 1958. Seeds from this sample were used in experiments during April 1959.

Seeds were planted by means of a suction counter on wetted germination blotters in plastic boxes (12 cm sq), 100 seeds to each box. Immediately each box was placed between folds of black cloth on a tray at a controlled temperature. After a definite number of hours in darkness the imbibed seeds were taken to the exposure room, irradiated, again wrapped in black cloth, and returned to a controlled temperature.

The boxes containing seeds were removed from the darkness only for irradiations of definite durations. Red light was obtained by filtering the light from a bank of 2 or 18 96-inch T8 slimline cool-white fluorescent tubes through two layers of red cellophane. Far-red irradiation was obtained by filtering the light from three 300-watt internal-reflector incandescent-filament lamps through two layers of red and of blue cellophane and about 6 cm of water. A specified exposure to either light source was made at a distance of 1.1 m. At the level of the irradiated seeds the intensity in the region 5,800 to 6,950 A was 700 and 6,000 ergs/cm²/sec for 2 and 18 tubes, respectively, and in the region 6,950 to 7,900 A was 7,500 ergs/cm²/sec.

After irradiation the seeds were returned to a controlled temperature for 7 to 21 days before the germinated ones were counted. Results are given as percent germination of the sound seeds (total seeds minus empty & dead seeds); per cent germination of the sound, light-requiring seeds; or per cent inhibition of red-light-promoted seeds. Per cent germination of sound seeds expresses the results in terms of the seeds that germinate divided by the total sound seeds. Per cent germination of sound, light-requiring seeds expresses the results in terms of the sound seeds that do not germinate in the dark:

$$\frac{\text{Total germinated seeds} - \text{dark-germinated seeds}}{\text{Total sound seeds} - \text{dark-germinated seeds}} \times 100$$

Per cent inhibition refers to the red-light-promoted seeds that do not germinate after exposure to far-red:

$$\frac{\text{Total seeds germinated after red saturation} - \text{seeds germinated after far-red}}{\text{Total seeds germinated after red saturation}} \times 100$$

Transverse sections of 200 seeds from each seed lot indicated the proportions of empty, discolored, and sound seeds.

RESULTS

OPTIMUM TEMPERATURE: The optimum temperature for conducting the tests was determined. Four boxes of seeds from sample LP-13 were used for each of the nine temperature conditions shown in table I. After approximately 28 hours in darkness, two boxes of imbibed seeds from each temperature were exposed 60 minutes to red (6,000 ergs/cm²/sec)

[1] Received October 12, 1960.

TABLE I

EFFECT OF TEMPERATURE WITH* & WITHOUT LIGHT ON GERMINATION OF PINUS VIRGINIANA SEEDS (LP-13)

TEMPERATURE °C DURING:		% GERMINATION OF SOUND SEEDS AFTER:	
		IRRADIATION WITH RED (6,000 ergs/cm²/sec)	
28-hr IMBIBITION	GERMINATION	0 min	60 min
10	10	0	0
15	15	0	0
20	20	0	8
25	25	1	62
30	30	2	8
5	25	< 1	92
10	30	2	39
20	30	< 1	20
30	20	0	18

* Irradiation after 28-hour imbibition.

radiation and the other two were maintained as dark controls. Seeds germinated at 10° or 15° C were counted after 21 days and those at other temperatures after 10 days. Less than 5 % of the seeds germinated in darkness at any temperature tested. Seeds exposed 60 minutes to red light germinated very well at certain temperatures, but not at others. Seeds germinated best (92 %) when imbibed at 5° and germinated at 25°.

RED, FAR-RED ENERGIES: Germination responses at 25° C of *Pinus virginiana* seeds (LP-13) held 22 hours in darkness at 5° to increasing irradiations with red (700 ergs/cm²/sec) and far-red (7,500 ergs/cm²/sec) were determined. The germinated seeds were counted 7 days after irradiation. The percentage promotion (table II) ranged from 2 % for a half minute to a maximum of 82 % for 32 minutes. After irradiation of the seeds by red for 64 minutes, 16 minutes of far-red inhibited germination of almost all the promoted seeds. Far-red for 1 minute did not inhibit germination. Measurements of the incident energies used and calculations, according to Hendricks et al (2), of the percentage pigment conversion for a given germination (fig 1) indicated that approximately 1×10^6 ergs/cm² was required for 50 % conversion of the pigment in either direction.

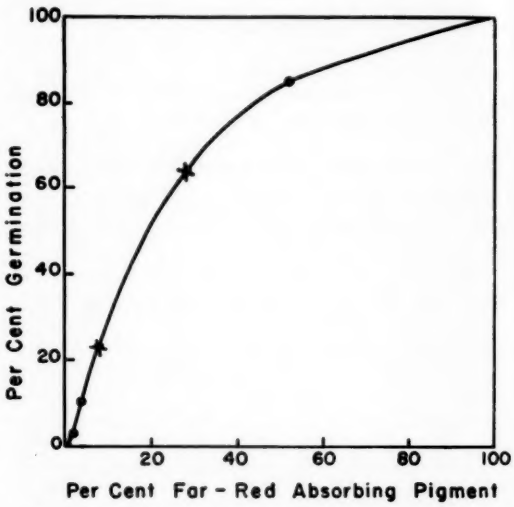

FIG. 1. Variation in the percentage germination of *Pinus virginiana* seeds (sample LP-13) with the amount of the controlling pigment in the far-red absorbing form. X indicates the two points of the experimental data chosen for calculation of the curve.

TABLE II

EFFECT OF INCREASING IRRADIATIONS OF RED (700 ergs/cm²/sec) & FAR-RED (7,500 ergs/cm²/sec) FOLLOWING A 22-HOUR IMBIBITION IN DARKNESS AT 5° C ON GERMINATION AT 25° OF PINUS VIRGINIANA SEEDS (SAMPLE LP-13)

MIN OF IRRADIATION WITH RED OR FAR-RED	% GERMINATION OF SOUND LIGHT-REQUIRING SEEDS AFTER PROMOTION BY RED*	% INHIBITION BY FAR-RED OF SEEDS PROMOTED BY 64 MIN OF RED
½	2	...
1	8	0
2	21	3
4	31	34
8	51	77
16	68	95
32	82	96
64	82	98

* Seeds germinated 1 % in total darkness.

TABLE III

EFFECT OF DURATION & TEMPERATURE OF IMBIBITION ON GERMINATION RESPONSES AT 25° C OF PINUS VIRGINIANA SEEDS (LP–64) TO INCREASING IRRADIATIONS OF RED (700 ergs/cm²/sec) & FAR-RED (7,500 ergs/cm²/sec)

MIN OF IRRADIATION WITH RED OR FAR-RED	% GERMINATION OF SOUND SEEDS AFTER IRRADIATION WITH RED WHEN IMBIBED AT:				% INHIBITION BY FAR-RED OF SEEDS PROMOTED BY 64 MIN OF RED WHEN IMBIBED AT:			
	25° C		5° C		25° C		5° C	
	1 day	16 days	1 day	16 days	1 day	16 days	1 day	16 days
¼	2	0	4	21				
½	> 1	2	3	29				
1	6	3	12	34	14	6	12	2
2	11	2	24	65	39	25	25	4
4	23	11	40	74	84	59	51	18
8	26	22	61	80	95	83	83	28
16	55	49	65	89	95	93	95	79
64	67	66	81	90	97	94	94	89

IMBIBITION TEMPERATURE: The temperature during imbibition controlled the total number of seeds that germinated in response to a saturating light stimulus. Seeds imbibed in darkness at 5° C or 25° and held at these temperatures for 1 or 16 days were given increasing irradiations with red (700 ergs/cm²/sec) and far-red (7,500 ergs/cm²/sec) and then placed in darkness at 25°. After 7 days the germinated seeds were counted (table III). A 64-minute exposure promoted 80 to 90 % of the seeds imbibed at 5° but about 65 % of those imbibed at 25°. Seeds imbibed at 25° were very easy to inhibit. Seeds held for the longer period at 5° were easier to promote and more difficult to inhibit than those held for only one day at the lower temperature.

IMBIBITION PERIOD: Lengthening the imbibition period in darkness at 5° C from 1 to 16 days increased sensitivity of seeds to red light. Seeds held at 5° for 1, 2, 4, 8, and 16 days were given increasing irradiations with red (6,000 ergs/cm²/sec) and far-red (7,500 ergs/cm²/sec) and then placed in darkness at 25°. Germination in total darkness was only slightly higher with the longer periods of imbibition for seeds of sample LP-62, but was markedly higher for those of sample LP-63 (table IV). Thus the population of seeds in the latter sample on which one could test light effects was small for the longest period of imbibition (table V).

TABLE IV

EFFECT OF PERIOD OF IMBIBITION AT 5° C ON SUBSEQUENT GERMINATION OF PINUS VIRGINIANA SEEDS IN TOTAL DARKNESS AT 25°

SAMPLE NO.	% GERMINATION IN DARKNESS OF SOUND SEEDS AFTER INDICATED PERIODS OF IMBIBITION				
	1 day	2 days	4 days	8 days	16 days
LP–62	4	11	12	7	16
LP–63	4	9	27	42	66

The energy of red light required for a given germination decreased as the period at 5° C increased (table V). As the seeds changed during dark imbibition so that they required less red light for promotion, they also changed so that they required more far-red radiant energy for inhibition (tables V & VI).

RED, FAR-RED REVERSIBILITY: The action of the red and the far-red radiation on the germination of pine seeds was immediately and repeatedly reversible (table VII). Twelve lots of 100 seeds were planted and held 96 hours in darkness at 5° C. Two lots were kept as dark controls and the remaining ones were exposed to red (6,000 ergs/cm²/sec) for 8 minutes. Two of these lots were immediately put under black cloth and the remaining eight were exposed 16 minutes to far-red (7,500 ergs/cm²/sec) and two of these were placed under black cloth. Alternating treatments with red and far-red, with removal of two lots to darkness after each operation, were continued until all lots were used. The last two lots thus received three red treatments alternated with two far-red treatments. The germinated seeds were counted 7 days after irradiation. When the exposure to red was last, approximately 90 % of the seeds germinated. When far-red was last the promoted seeds and even some of the dark germinators failed to germinate. Almost identical results were obtained on two samples.

RATE OF GERMINATION PROCESSES: When light-requiring seeds are exposed to red light the pigment is converted to the far-red-absorbing form, the active form of the pigment. With the pigment in this form the seeds progress towards germination. The ability to prevent the promoted seeds from germinating by far-red radiant energy depends on the interval of time

TABLE V

EFFECT OF INCREASING IRRADIATIONS OF RED AFTER VARIOUS PERIODS OF IMBIBITION AT 5° C ON GERMINATION AT 25° OF PINUS VIRGINIANA SEEDS

SAMPLE NO. & IMBIBITION PERIOD	% SOUND SEEDS REQUIRING LIGHT FOR GERMINATION	% LIGHT-REQUIRING SEEDS GERMINATING AFTER IRRADIATION (6,000 ergs/cm²/sec) FOR:					
		1 min	2 min	4 min	8 min	16 min	32 min
LP–62:							
1 day	96	46	60	79	82	78	77
2 days	89	54	62	73	83	73	86
4 days	88	67	84	89	90	92	91
8 days	93	75	77	83	96	91	95
16 days	84	83	81	86	89	89	90
LP–63:							
1 day	96	43	44	57	69	70	67
2 days	91	60	69	68	88	82	89
4 days	73	81	83	91	94	93	94
8 days	58	87	87	94	91	96	95
16 days	34	86	88	98	88	91	92

between the two irradiations. The period that the pigment must be kept in the far-red-absorbing form before the processes leading to germination can no longer be stopped by far-red was measured. Seeds of *Pinus virginiana* held in darkness at 5° C for 1 or 20 days were exposed 16 minutes to red (6,000 ergs/cm²/sec). These promoted seeds were held in darkness at 25° for 0, 8, 16, 24, 48, 72, 96, and 120 hours and then given 16 minutes of far-red (7,500 ergs/cm²/sec). The seeds were returned to darkness after the far-red treatments and the germinated seeds counted after 9 days. Inhibition of 50 % of the promoted seeds in lots imbibed 1 day resulted from far-red treatments given 48 to 72 hours after the red but for lots imbibed 20 days the corresponding periods were 8 to 16 hours (table VIII).

DISCUSSION

The germination response of *Pinus virginiana* seeds to light is basically the same as that of other seed kinds (3, 5). The photoreaction is controlled by the red, far-red pigment system. It is immediately and repeatedly reversible. And, like other seeds, *Pinus virginiana* seeds become more sensitive to red when they become less sensitive to far-red and vice versa.

TABLE VI

EFFECT OF INCREASING FAR-RED IRRADIATIONS AFTER VARIOUS PERIODS OF IMBIBITION AT 5° C ON GERMINATION AT 25° OF RED LIGHT PROMOTED* PINUS VIRGINIANA SEEDS

SAMPLE NO. & IMBIBITION PERIOD	% SOUND SEEDS PROMOTED BY RED LIGHT	% INHIBITION OF RED LIGHT PROMOTED SEEDS AFTER FAR-RED IRRADIATIONS (7,500 ergs/cm²/sec) FOR:				
		2 min	4 min	8 min	16 min	32 min
LP–62:						
1 day	77	7	29	81	94	94
2 days	86	12	43	83	91	93
4 days	91	8	10	19	82	83
8 days	95	10	13	50	80	83
16 days	90	1	3	23	63	75
LP–63:						
1 day	67	36	75	93	99	98
2 days	89	26	62	91	98	99
4 days	94	8	33	83	90	97
8 days	95	0	10	55	90	92
16 days	92	0	2	32	60	68

*32 minutes red (6000 ergs/cm²/sec) after days of imbibition and prior to far-red radiations.

TABLE VII

EFFECT OF ALTERNATE RED & FAR-RED IRRADIATIONS ON GERMINATION AT 25° C OF PINUS VIRGINIANA SEEDS IMBIBED 96 HOURS AT 5° IN DARKNESS

CHARACTER OF IRRADIATIONS*	% GERMINATION OF VIABLE SEEDS IN	
	LP–62	LP–63
0 (dark control)	4	14
R	92	89
R + FR	4	2
R + FR + R	94	93
R + FR + R + FR	3	1
R + FR + R + FR + R	93	92

* Red, 6,000 ergs/cm²/sec, 8 minutes; far red, 7,500 ergs/cm²/sec, 16 minutes.

Pinus virginiana seeds required ten times the energy needed by Lepidium seeds for 50 % conversion of the far-red-absorbing form of the pigment to the red-absorbing form. And they required about five times the energy for 50 % conversion in the opposite direction. The greater incident energy requirement of *Pinus virginiana* seeds probably results from the greater thickness of their seed coats, which consequently transmit less energy than seeds of Lepidium.

Pine seeds become more sensitive to red and less sensitive to far-red radiant energy as the imbibition period at 5° C is increased from 1 to 16 days. The light requirement of many seeds for promotion of germination to a given percentage often varies as a function of temperature or duration of imbibition. The far-red energy for a given percentage inhibition almost invariably changes in the opposite direction. Hitherto, the explanation for the greater sensitivity to red of these pine seeds would have been that the longer periods were effective in changing a reactant for conversion of the pigment into a more oxidized form. A more likely explanation comes from recent studies on the extracted pigment. These studies (1) show that the percentage conversion depends on the energy of irradiation. Moreover, dialysis of the pigment solution or the addition of oxidizing or reducing agents does not inactivate the photoreaction. Thus indications are that the inverse change of sensitivities depends on the degree of pigment conversion required for a given germination rather than an associated reactant.

The germination processes proceed at a faster rate for seeds promoted with red light after a 20-day imbibition period at 5° C than for seeds promoted after a 1-day imbibition period. A longer time can elapse between red and far-red radiations for seeds held 1 day at 5° than for those held 20 days and still prevent 50 % of the promoted seeds from germinating. The pigment was already converted to the germinating form in all these seeds. The difference, therefore, in rate of germination is due to some factor other than photoreaction. Also the fact that the number of seeds that germinated in total darkness increased with longer periods at 5° showed that changes (after-ripening) occurred in these seeds while they were held at the low temperatures.

The interaction of red light treatment with period of imbibition at a low temperature (stratification) permits shortening of the stratification period required to induce high germination.

The four seed lots, collected in different years and stored differently, showed differences in the degree of response to light and temperature. Because the samples varied by more than one characteristic, it was not possible to relate the responses to effects of age or storage conditions.

TABLE VIII

RATE OF ESCAPE FROM CONTROL BY FAR-RED RADIANT ENERGY OF PROMOTED SEEDS OF 2 SAMPLES OF PINUS VIRGINIANA IMBIBED AT 5° C FOR 1 OR 20 DAYS & GERMINATED AT 25°

HOURS OF DARKNESS BETWEEN RED & FAR-RED*	% INHIBITION OF RED LIGHT PROMOTED SEEDS IMBIBED FOR:			
	1-DAY		20-DAYS	
	LP–62	LP–63	LP–62	LP–63
0	90	99	69	61
8	91	99	89	68
16	80	95	73	33
24	73	91	37	11
48	43	65	8	0
72	13	30	4	< 1
96	5	0	...	...
120	2	0	...	...

* Red, 6,000 ergs/cm²/sec, 16 minutes; far-red, 7,500 ergs/cm²/sec, 16 minutes.

SUMMARY

Seeds of *Pinus virginiana* Mill. imbibed at different temperatures for various periods were tested for their response to red and far-red radiant energies.

Very little germination occurred in darkness at any temperature tested unless the seeds were held at 5° C for a period prior to placing them at the germinating temperature. With light, an imbibition temperature of 5° followed by a germination temperature of 25° was most favorable.

Germination of the seeds was promoted by red and inhibited by far-red radiant energy. About 1×10^6 ergs/cm² of energy was needed for conversion of 50 % of the pigment by red or far-red radiant energy.

The maximum germination of seeds in response to red light was greater if they were imbibed at 5° C than at 25°. Lengthening the period of imbibition at 5° increased germination in total darkness—very slightly for one sample and markedly for another. Lengthening the period of imbibition at 5° increased

the sensitivity of both lots of seeds to promotion by red.

The action of the red and far-red radiant energies on the germination of the seeds was immediately and repeatedly reversible.

After seeds were imbibed for 20 days before exposure to red light the processes of germination progressed much faster than in those seeds imbibed 1 day. Thus far-red could affect 1-day-imbibed seeds for longer periods after exposure to red than 20-day-imbibed seeds.

Acknowledgment

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UPTAKE OF MAGNESIUM & ITS INTERACTION WITH CALCIUM IN EXCISED BARLEY ROOTS[1,2]

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Very often Ca and Mg are considered as similar ions with respect to their absorption by higher plants. No doubt, this attitude is based on the behavior of the ions in greenhouse and field studies, as well as upon their chemical similarities. For instance, Collander (3) concluded from a greenhouse study that, in general, Ca and Mg were absorbed in about equal amounts. Overstreet and Jacobson (20) point out in their review that Ca and Mg are slowly absorbed ions compared to the monovalent cations.

Almost no work has been done on the absorption of Mg by higher plants under controlled laboratory conditions. Handley and Overstreet (7) report that Ca and Mg have little influence on the respiration of excised barley roots, presumably because they are slowly absorbed. However, the absorption of Mg was not actually determined in their study.

Although Ca and Mg are generally grouped together as being slowly absorbed ions, there is little or no direct experimental evidence to warrant this conclusion. Most, if not all, of the Ca uptake by excised barley roots in the so-called physiological pH range was found to be non-metabolic (18). Recently, Conway and Beary (4) and Rothstein, et al (23) have reported Mg to be rapidly absorbed by yeast from single salt solution. In light of the latter findings, the uptake of Mg by excised barley roots was investigated.

Materials & Methods

All of the experiments were conducted using 6-day-old excised barley roots of the variety Tennessee Winter, 1956 crop. The method of culturing the root material was essentially that of Jacobson, et al (13). The excised roots were washed several times in distilled water prior to their use. After washing, the roots were centrifuged at 65 $\times$ g for 5 minutes. Representative samples of roots were weighed out and placed in bottles containing the desired salt solutions kept at constant temperature with suitable aeration. All experiments were conducted at 25° C unless otherwise indicated. The pH of the solutions

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was rigorously controlled by additions of acid or base throughout the experimental period. Seven grams of roots in 7 liters of solution were used in all experiments. Changes in chemical composition of the solutions due to uptake or pH adjustments during the absorption period were negligible.

Stock solutions of $Mg(OH)_2$ were prepared by allowing saturated solutions of this base to stand in closed pyrex bottles for several weeks. Just prior to the experiments, portions of the clear supernatant solution were siphoned off. Care was taken to avoid contact of the $Mg(OH)_2$ with CO_2 of the air.

At the end of the experimental period, the roots were separated from the solutions on a fine mesh nylon screen. The roots were washed in a running stream of distilled water for 10 seconds. The washing procedure was standardized to minimize variations in the non-metabolic uptake component. Roots were dried in an oven overnight and ashed in a muffle furnace at 600° C. Potassium was determined by flame photometry on an acidified solution of the ash. Aliquots of these same solutions were titrated with ethylenediamine tetraacetic acid (EDTA) to the Eriochrome Black T endpoint for Ca plus Mg (2). Ca was determined by EDTA titration to the Calcein endpoint (5) and Mg was obtained by difference. Root samples for organic acid analysis were frozen rapidly with liquid nitrogen at the end of the experiment. The material was lyophylized and organic acids were determined by ether extraction and titration (22). Inorganic ions were determined on ashed samples of the lyophylized materials as described above.

Uptake data are expressed in meq per kilogram of fresh roots. These values represent the net change in content of the roots during the absorption period. Negative values indicate a loss of ions from the root. Initial contents of experimental material were 6.0 to 9.5 meq Mg, 11.2 to 13.4 meq K, and 2.2 to 2.9 meq Ca.

RESULTS

The first experiment was designed to determine the effect of pH on the uptake of Mg. Solutions of $Mg(OH)_2$ and $MgBr_2$ were mixed so that the resulting solutions contained 5 meq Mg per liter at pH 10. A pH range of from 2 to 10 was obtained by adjustment of these solutions with HBr. The pH of each solution was maintained throughout the 3-hour absorption period.

The large uptake of Mg in the physiological pH range (fig 1) is rather surprising in light of the largely non-metabolic nature of Ca uptake by this barley root material (18). These results are very similar to those reported by Conway and Beary for Mg uptake by yeast as a function of pH (4). It is also interesting to note the striking similarity between the absorption of Mg and the alkali cations. For excised barley roots, the rate of Mg absorption is approximately equal to that of Li and Cs and about half the rate for Na, K, and Rb (10). Furthermore, the shape of the pH curve is quite similar to those published by Jacobson, et al (12) for K.

It is highly unlikely that a Mg uptake of this magnitude was non-metabolic. However, to establish this fact an experiment was conducted to determine the effect of 2,4-dinitrophenol (DNP) and low temperature on Mg uptake at pH 5. In a parallel experiment, K uptake was used as a measure of the effectiveness of these treatments in reducing the metabolic absorption. The absorption period was 3 hours and the concentration of $MgBr_2$ or KBr was 5 meq per liter. For the treatments at low temperature, the roots were submerged in distilled water at 0° C for 1 hour prior to the uptake period to eliminate any

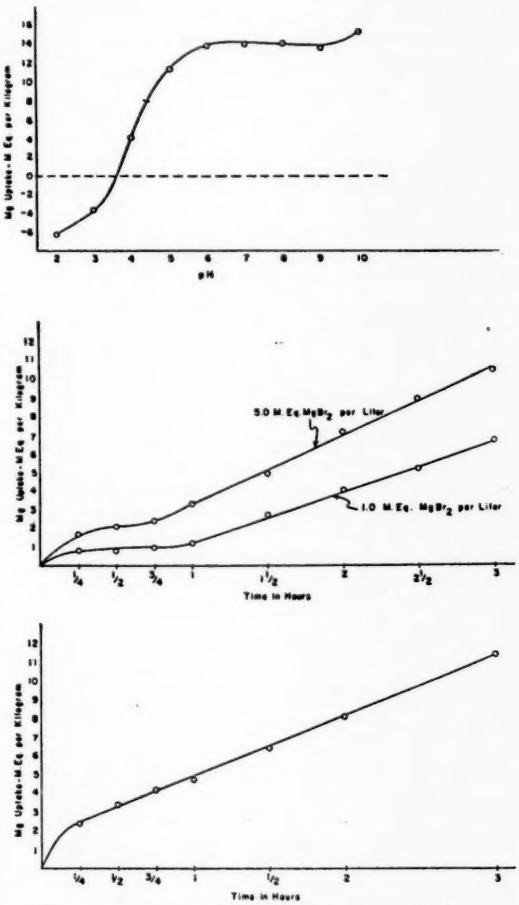


FIG. 1. The effect of pH on the uptake of Mg in 3 hours from 0.005 N $MgBr_2$.

FIG. 2. The uptake of Mg as a function of time from two concentrations of $MgBr_2$ at pH 5.

FIG. 3. Uptake of Mg as a function of time from 0.005 N $MgBr_2$ at pH 5. Roots were pretreated for 30 minutes in HBr at pH 4.

major residual metabolic activity carried over from room temperature (16). The control and DNP treatments were at 25° C. The data presented in table I clearly indicate that Mg is absorbed metabolically by excised barley roots.

As additional evidence of the metabolic absorption of Mg, an experiment was conducted to determine the ability of the barley root to balance excess Mg absorption by the production of organic acid anions (9). Roots were placed in a solution of 0.005 N $MgSO_4$ at pH 5 for 3 hours. Since sulfate is a slowly absorbed ion, excess cation absorption was expected to occur under these conditions (11). The results showed a Mg uptake of 7.9 meq per kilogram and an organic acid increase of 6.6 meq per kilogram. It is evident that over and above any non-metabolic uptake, excess Mg absorption and organic acid formation were closely related. This certainly places Mg in the category of a metabolically absorbed cation. Here again is a striking example of the similarity of Mg in uptake to K.

Time curves are often used in determining the steady-state absorption rate. The magnitude and duration of the initial portion of such a curve have been given considerable attention in the assessment of non-metabolic uptake (15). The absorption of Mg as a function of time at two concentrations of $MgBr_2$ is shown in figure 2. The absorption period varied from 15 minutes to 3 hours and the pH was maintained at 5. It is interesting to note the shape of the time curve at both concentrations. In each case there appeared to be a period of lag before the onset of the straight-line, steady-state absorption phase. This lag phase was slightly more pronounced at the lower concentration.

The root material used in these experiments was grown in a solution containing Mg. It seemed unreasonable, therefore, to assume that the lag phase observed in the time curves was due to an induction period for Mg absorption. A more plausible explanation is that the lag phase reflected a readjustment in the initial Mg of the root upon being placed into a single salt solution, even though it was a solution of $MgBr_2$. In other words, the uptake as a function of time may have been a composite curve of two processes: an uptake and a loss. Figure 3 shows the effect of a dilute acid pretreatment of the roots on the subsequent time curve for Mg uptake. The roots were pretreated for 30 minutes in HBr at pH 4. During the pretreatment, the Mg content decreased from 8.3 to 7.0 meq per kilogram. The subsequent Mg uptake was from a solution containing 5 meq $MgBr_2$ per liter at pH 5. This curve is directly comparable to the 5 meq curve shown in figure 2, since both were done at the same time on the same lot of root material. It is apparent from figure 3 that the lag phase has been eliminated or greatly reduced by the dilute acid pretreatment. The steady-state absorption rate does not appear to have been materially affected. The greater total uptake in 3 hours by the pretreated roots was due almost entirely to the different initial content of these roots compared with the untreated ones. These findings suggest that there is a labile fraction of initial Mg which is easily lost from the root. This is supported by a large number of observations on this root material which show a consistent loss of initial Mg to Ca and K solutions. The losses range from 1.2 to 4.0 meq per kilogram depending on the treatment. For the most part, the loss is complete in the first 30 to 45 minutes.

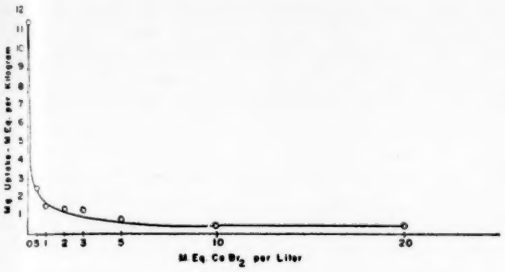
Table I

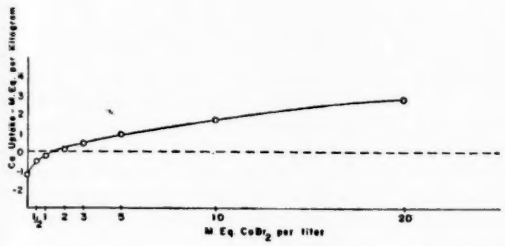
Effect of Low Temperature & DNP on Uptake of K & Mg

Treatment	Test solution	Cation uptake meq/kg 3 hr at pH 5
Control	KBr	23.5
0° C	KBr	3.0
10^{-6} M DNP	KBr	10.7
5×10^{-6} M DNP	KBr	0.9
10^{-5} M DNP	KBr	−0.4
Control	$MgBr_2$	12.5
0° C	$MgBr_2$	2.2
10^{-6} M DNP	$MgBr_2$	8.0
5×10^{-6} M DNP	$MgBr_2$	2.3
10^{-5} M DNP	$MgBr_2$	1.5

Ca has been shown to have a marked influence on the absorption of the alkali cations. Depending on the ion and the pH, Ca may either stimulate or depress the uptake (10). In view of the similarity between the absorption of Mg and that of the alkali cations, an experiment was conducted to determine the effect of Ca on Mg uptake. The absorption period was 3 hours and the pH was maintained at 5. For all treatments 0.005 N $MgBr_2$ solutions were used. The $CaBr_2$ concentration of these solutions varied from 0 to 20 meq per liter. It is immediately apparent from figure 4 that Ca markedly depressed the uptake of Mg. As little as 0.5 meq of Ca was effective in reducing the uptake by about 80 %. At equivalent concentrations of Ca and Mg the Mg uptake was reduced by almost 95 %. The Mg uptake at Ca concentrations above 3 meq per liter was within the accepted range of non-metabolic uptake for excised barley roots (9). However, it is impossible to say how much of the Mg uptake in this experiment was non-metabolic. In addition to the possible loss of a labile fraction of initial Mg, there is the additional complication of not knowing the cation exchange properties of the root surface in relation to Ca and Mg.

Figure 5 shows the uptake of Ca for the experiment described above. The data are entirely consistent with the hypothesis that there is little or no metabolic absorption of Ca by this root material (18). The interesting point, however, was the loss of Ca from the root at the lower Ca concentrations. Even though Ca was lost from the root, it was at these





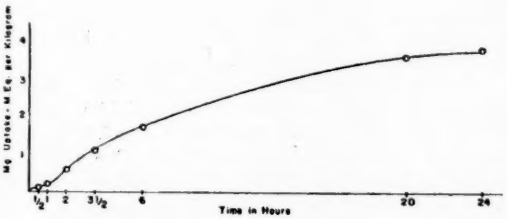


FIG. 4. The effect of various $CaBr_2$ concentrations on the uptake of Mg in 3 hours at pH 5. The $MgBr_2$ concentration was 0.005 N for all treatments.

FIG. 5. The uptake of Ca as a function of $CaBr_2$ concentration in the presence of $MgBr_2$. Experimental conditions are the same as for figure 4.

FIG. 6. The uptake of Mg as a function of time in the presence of Ca. $MgBr_2$ and $CaBr_2$ were each 0.005 N and the pH was maintained at 5.

lower concentrations that Ca was very effective in blocking Mg absorption.

Because of the uncertainty involved in assessing the non-metabolic uptake of Mg, it is difficult to determine the completeness with which Ca blocks the metabolic absorption of Mg. To gain a measure of this, a determination of the uptake of Mg as a function of time in the presence of Ca was made. It was anticipated that the Mg absorption rate would be quite small, therefore, time periods up to 24 hours were used. The concentrations of $CaBr_2$ and $MgBr_2$ were each 5 meq per liter. The pH was maintained at 5 throughout the experiment. The results, (fig 6) indicate that, although Ca was very effective in blocking Mg absorption, the blocking action was not quite complete. It is clear, however, that Mg was only slowly absorbed in the presence of Ca.

The marked effect of small amounts of Ca on Mg absorption suggested the possibility that the initial Ca content might be responsible for the lag phase observed in the Mg time curves (fig 2). The root material had been grown in a solution containing 0.2 meq Ca per liter. Thus, it could be argued that the lag phase represented a carry-over effect of Ca on Mg absorption. To check this possibility, a batch of roots was divided into three portions. One portion was left untreated and served as a control. The second portion was treated for 30 minutes in dilute HBr at pH 4. A third portion was treated in HBr at pH 4 for 30 minutes followed by a 30 minute treatment in 0.005 N $CaBr_2$ at pH 5. Samples of these three portions were placed in 1 meq $MgBr_2$ per liter at pH 5 for various time periods. The untreated control roots showed a lag phase similar to the 1 meq time curve (fig 2). Neither portion of the pretreated roots exhibited a lag phase. Furthermore, the steady-state rate of Mg absorption was not influenced by the pretreatment. On the basis of these results, it appears that a Ca pretreatment has no effect on the subsequent Mg absorption. Therefore, the initial Ca content of the root was probably not responsible for the lag in the time curve for Mg uptake.

DISCUSSION

The existence of a labile fraction of Mg, as postulated here, makes it virtually impossible to select a value for the initial Mg status of these roots. Consequently, a non-metabolic uptake determination for Mg can not be made with any certainty. Therefore, no attempt has been made to apply a non-metabolic uptake correction to the data reported in this paper. It should be pointed out, however, that a non-metabolic uptake correction would in no way alter the conclusions drawn from this study.

The effect of Ca on the permeability of cells to electrolytes has been recognized for a long time. Much of the early work dealt with the antagonistic effects of Ca on many ions. The conclusion was drawn that Ca markedly decreases the permeability of cells (17, 19, 24). A general decrease in permeability, however, would imply that the movement of all ions into the cell is uniformly depressed. This does not appear to be the case, at least for excised barley roots. As has been shown here, Ca markedly decreases the uptake of Mg. Also, it has been shown elsewhere that Ca almost completely blocks the uptake of Li and probably has a similar effect on H ion (10). Contrasted with these results, Ca either stimulated or had little effect on the absorption of K, Rb, and Cs from acid solutions. The effect of Ca on Na absorption is intermediate between these two extremes. It would seem therefore, that the action of Ca is to enhance the permselectivity of the cell rather than to effect generally its permeability.

The overall ion absorption process has been en-

visioned as consisting of at least two steps (10). The first of these is the movement of the ions through a permselective region at the cell surface. The second step is the actual metabolic accumulation of ions and occurs at some point removed from step 1. The permselective properties of the cell surface region are regulated by Ca and determine the kind and amount of ions reaching the metabolic absorption site. The results reported here on the effect of Ca on Mg absorption are entirely consistent with this hypothesis. Just as with Ca-Li relationships (10), it is impossible to explain the effect of Ca on Mg as being simply a mutual competition for the same binding site. When two ions mutually compete, each ion has a depressing effect on the metabolic absorption of the other. For the barley roots used in these experiments, metabolic Ca absorption was found to be either completely absent or too small to be measured by the techniques used (18). It is difficult to see how Ca could be competing with Mg for binding sites under these circumstances in a fashion similar to that suggested for Ca-Li (6). Furthermore, in experiments not reported here, increasing the Mg concentration in the presence of Ca did not overcome the blocking action of Ca on Mg uptake. Even at ratios as high as 20 meq Mg per liter to 1 meq Ca per liter, the blocking action was still quite pronounced. If the two ions were competing for the same binding site, at least a partial reversal in the Ca effect would be expected. It is concluded, therefore, that the action of Ca is at some point other than at the binding site.

The evidence for the action of Ca being on the surface of the cell has been discussed previously (18). The failure of the Ca pretreatment to influence the subsequent Mg absorption rate is additional evidence to support this view. Also, the lack of a carry-over effect from the Ca pretreatment indicates that the active Ca is only weakly held on the surface. The enhanced permselective properties of the cell are apparently established at low Ca concentrations and maintained over a wide range of Mg to Ca ratios. As was pointed out above, a marked blocking action was observed at a Mg: Ca ratio of 20: 1. This fact would seem to eliminate the possibility that the active Ca is occupying strictly cation exchange sites. Perhaps Ca enters into chemical combination with groups in the cell surface but is easily hydrolyzed off in a Ca-free solution.

Not all of the Ca associated with the root is active in blocking the absorption of Mg. This is illustrated by the data presented in figures 4 and 5. At the lower Ca concentrations, the blocking action is quite evident even though there is a net loss of Ca from the root. At least a portion of this non-active Ca may be associated with pectic substances in the cell wall.

The mechanism whereby Ca enhances the permselectivity of the cell surface is a matter of speculation at this time. Ca may act by altering the structural configuration of the surface groups in such a way as to markedly change their chemical and physical properties. It has been postulated that Ca protects the cell membrane from the action of ribonuclease (8) and basic redox dyes (21). Ca does not appear to be unique in this role, since other polyvalent cations may also behave similarly. However, Ca may be the most important polyvalent cation in this respect, since it is not absorbed rapidly and is apparently not toxic over long periods of time. In addition, Ca is almost always the most dominant polyvalent cation present in the natural root environment.

Extrapolation of the steady-state portion of the two time curves in figure 2 back toward the origin reveals that they both cross the ordinate at negative uptake values. Results like these point to the danger of extrapolation of time curves as a measure of non-metabolic uptake as has been done by some workers (1, 14). Even when the extrapolation seems reasonable (i.e., when it cuts the ordinate at positive uptake values), there is no assurance that the procedure is a valid one. Lag phases of shorter duration than those shown here may exist, even for ions other than Mg, and may well go unnoticed. For example, the time curves reported here have been repeated a number of times and while there was always a lag phase for the untreated root material, its duration and magnitude varied from experiment to experiment. In this way a range of intercept values, both positive and negative, has been observed.

Although the roots had been grown in a solution containing Mg, it is doubtful that the labile fraction which was lost represented non-metabolic or free space Mg from the culture solution. The root material for these experiments had been washed several times in distilled water before they were used. Furthermore, the concentration of Mg in the test solution was many fold higher than in the original culture solution. If the lag phase in the time curves does reflect a loss, the Mg must be coming from a compartment which is higher in concentration than even the test solution. Such a compartment could only be one into which the Mg had been previously taken up by metabolic means.

Summary

Mg was found to be rapidly absorbed by 6-day-old excised barley roots. The absorption of Mg was sensitive to both low temperature and dinitrophenol. In addition, excess Mg absorption was found to be related to organic acid production in the root. These facts establish the metabolic nature of Mg absorption. In general, the absorption of Mg from single salt solutions was more similar to the alkali cations than to Ca.

A lag phase was observed in the time curves for Mg uptake. The lag phase could be eliminated by a 30 minute pretreatment of the roots in a dilute acid solution. It is suggested that there is a labile fraction of the initial Mg content which is easily lost from the root, even to a solution which contains Mg.

A large fraction of the Mg absorption was blocked very effectively by small amounts of Ca. The blocking action was quite pronounced, even at Ca concentrations where there was a net loss of Ca from the root. It is proposed that Ca acts by altering the permselective properties of the cell surface region.

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TRANSLOCATION AND RE-USE OF BORON IN BROCCOLI

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The question of boron mobility and re-use in plants has not been adequately investigated. It is often dismissed with the statement that a continuous supply of boron is necessary during growth because boron cannot be stored and re-used. However, there is evidence that boron is not entirely immobile, although re-use has not been definitely established. Eaton, McCallum, and Mayhugh (3) showed that, under conditions of excess boron, stone fruit trees translocate boron to the fruit and that high concentrations can accumulate there while only moderate concentrations remain in the leaves. Scott and Schrader (8) showed that the concentration of boron in older leaves of the grape plant decreased after the source in the root media had been removed. They suggested that plants use stored boron in the formation of new tissue during the interval between omission of boron from the nutrient solution and the appearance of deficiency symptoms in the plant. Johnson and Fisher (6) performed such an experiment and found that tomato plants apparently grew normally for 3 weeks. Chandler (1) also suggested that boron is translocated from older leaves of various Brassica species and used in new growth during the period following the omission of boron from the nutrient solution. Shive (9), and Reeve and Shive (7) determined the percentage of insoluble boron in plant tissue in order to explain its immobility. Monocotyledons had a high percentage of soluble boron, even in plants deficient in this element, which may account for their low requirement, whereas dicotyledons had high proportions of insoluble boron. Eaton (2) found a large number of plants had essentially all of their boron in the sap. These studies suggest that some plants at least should be able to translocate boron from one tissue and re-use it in another.

The objective of this work was to evaluate the re-distribution of boron for re-use in the broccoli plant.

METHODS

Broccoli (*Brassica oleracca* Linn. var. botrytis DC.) (Hort. var. Puyallup Polycross 2) was grown in quartz gravel with several irrigations a day from a 150-gallon reservoir of Hoagland's nutrient solution no. 1 with the supplementary minor elements (5). Two plants were grown per 3-gallon crock and later thinned to one. At about 45 days before flowering the plant size was estimated by calipering the stem and counting the number of leaves. A randomized block experiment was established using plant size as a basis for grouping into blocks. The 12 largest plants became one group, the 12 smallest became another group, and two groups were established with the intermediate size plants. All 48 plants were numbered and assigned a harvest date so that one plant from each size group would be harvested each period. There were 12 harvest dates occurring at 4-day intervals. On the date designated for Period 1 the nutrient solution was dumped and the gravel was flushed four times with distilled water and nutrient without boron then provided. At the end of each of 12 assigned intervals, the plants of a particular group were cut at the cotyledonary node and the fresh weight was taken. The leaves were then stripped from the stem and numbered consecutively from the base of the plant. Leaves 1, 2, and 3 were combined into one sample. All other leaves and stem were dried at 65° C in a ventilated oven, weighed, and analyzed separately for total boron. Boron was determined by the method of Hatcher and Wilcox (4).

Leaves 1 to 6 inclusive became senile and abscissed during the course of the experiment. All dead leaves were collected and labeled according to the predetermined sampling date and were used to compute the total boron content of the plant (excluding roots). Only leaves alive at sampling were used to estimate boron movement. Leaves 1, 2, and 3 were alive through the fourth period, leaf 4 through the sixth period, leaf 5 through the eighth period, and leaf 6 through the tenth period. Leaves 7 to 14 inclusive were available for all 12 periods. Leaves 15 and younger were formed during the course of the experiment. The number of sampling dates for these leaves is indicated in the figures.

[1] Received October 24, 1960.

[2] Scientific Paper no. 1732 of the Washington Agricultural Experiment Stations. Project 335. Joint contribution of the Tree Fruit Experiment Station, Wenatchee, Wash., and the Horticultural Crops Research Branch, Agricultural Research Service, U. S. Department of Agriculture.

Regression Analysis

A regression of boron concentration (ppm) on time in a plant part will not indicate a net movement of boron from or to that part if the plant part changes in weight concomitantly. However, a change in total boron content of a plant part will indicate a net movement of boron, regardless of the weight change. A significant negative correlation of total boron with time will indicate a boron withdrawal. To illustrate boron movement in the broccoli plant, five separate regression equations were calculated for each leaf. These include: A, the change in dry weight of the leaf with time; B, change in boron concentration with time during periods 1 through 7; C, change in boron concentration with time during periods 7 through 12; D, change in total boron with time during periods 1 through 7; E, change in total boron with time during periods 7 through 12. The calculated regressions were all linear except "A", which was a parabolic section. For leaves 13 and younger, regression equations for the total boron were not calculated. For these leaves there was an obvious deviation from linearity which is adequately illustrated in figure 4.

Results

Symptoms of boron deficiency appeared on most plants at the seventh harvest date. Most of these symptoms have been adequately described previously (1), but are given here because the original source is out of print. The first symptom to appear is a wet or water-soaked area on the stem usually under one of the leaves near the top of the plant (fig 1A). As the deficiency becomes more severe, cracks develop

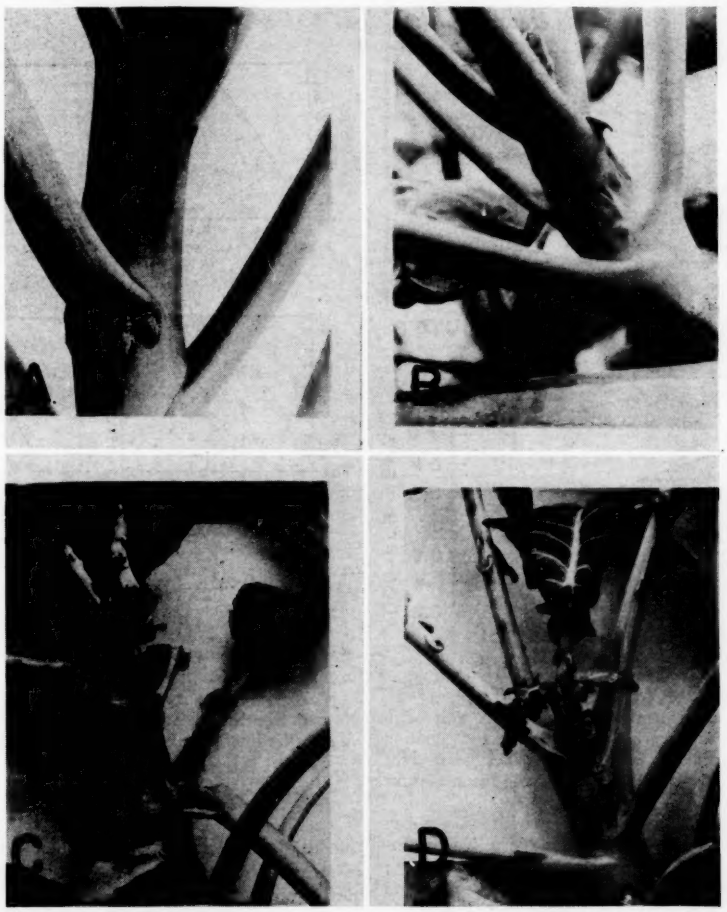

Fig. 1. Symptoms of boron deficiency in broccoli: A, The first symptom to develop is a water-soaked spot under one of the leaves. B, This spot later dries and cracks. C, The internal stem tissue develops corky areas. D, Later the tip becomes dwarfed, midribs curve downward, leaves roll under, and wart-like growths develop on leaf veins.

in this portion of the stem while the rest of the plant appears normal (fig 1B). The internal tissue of the stem develops dry or corky areas (fig 1C). Later the tip leaves become dwarfed, the midribs are curved downward, the edge of the blade rolls under, and small, wart-like growths appear on the leaf veins. Cracks develop in the entire upper portion of the stem (fig 1D).

The total fresh weight of the plant (excluding roots) increased logarithmically with time (fig 2) through the ninth harvest period (32 days). The increase thereafter also appeared to be logarithmic, but with slightly less slope and the curve is offset slightly to the right. This can be accounted for by the dying and drying of the older and lower leaves. There was some dwarfing of the youngest leaves after deficiency symptoms appeared, which will account for a slight change in slope of the growth curve. Because the lower leaves showed senility and were harvested to prevent their loss, the growth curve was calculated only through the first nine harvest periods.

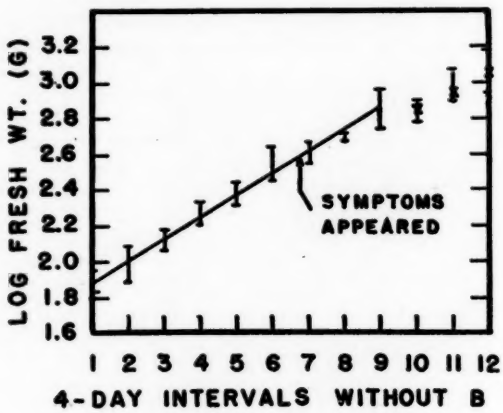

FIG. 2. Increase in fresh weight of broccoli with time. After converting fresh weight to logs, the equation $y = 1.77 + 0.120x$ and $r = 0.975$ describes the weight increase. For periods 10, 11, 12, "x" is the mean of four values.

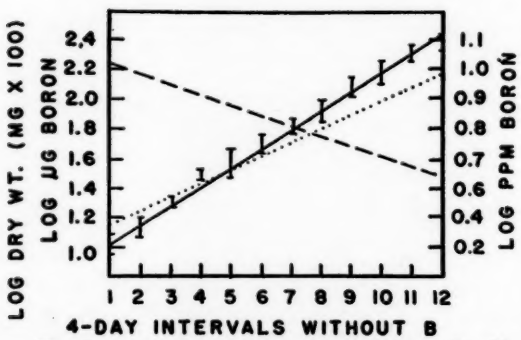

FIG. 3. Changes in broccoli stem with time when grown on boron-less nutrient solution: dry weight increase (*solid line with data indicated by vertical bars*); increase in total micrograms boron (*dotted line*); and decrease in boron concentration (ppm) (*broken line*). After converting data to logs, the equations and correlation coefficients are: Dry Weight: $y = 0.92 + 0.125x$, $r = 0.98^{**}$. Micrograms: $y = 1.07 + 0.917x$, $r = 0.95^{**}$. PPM: $y = 1.15 - 0.034x$, $r = 0.85^{**}$.

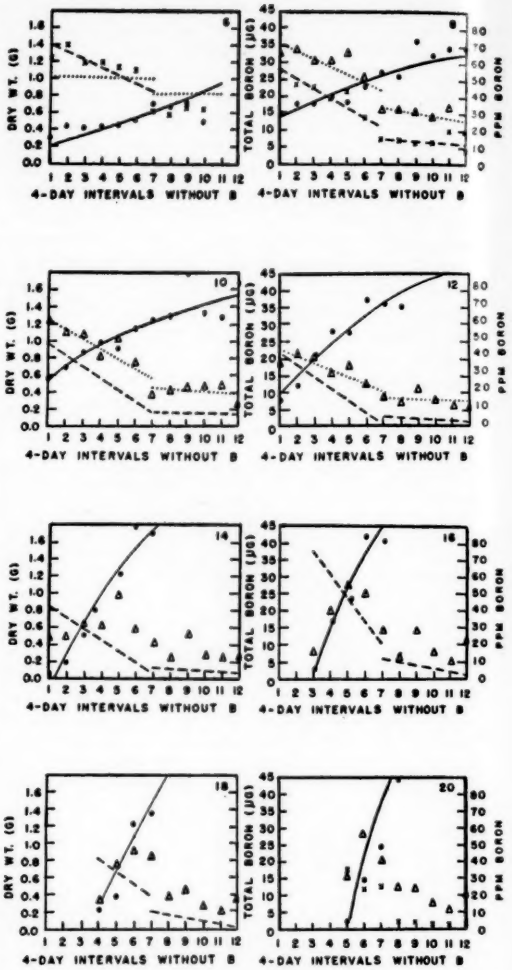

FIG. 4. Change in dry weight of leaf with time (*solid line with circular data points*), change in boron concentration (ppm) with time (*broken line and/or x data points*), and change in total boron content (micrograms per leaf) with time (*dotted line with triangular data points*) in broccoli leaves when grown in boron-less nutrient solution. Even numbered leaves 6 through 20 are shown. Leaf number is given in upper right hand corner of each figure. The legends on the ordinate refer to both figures in each pair. All curves are calculated regression lines; statistical significance is given in table I.

The broccoli plant continued to grow at a normal rate for a considerable time after deficiency symptoms appeared. This continued growth is better illustrated in figure 3 which shows the growth of the stem. There was little indication of any lessening in growth rate during the 17 days that these plants grew after deficiency symptoms first appeared.

The total boron content of the aerial part of the plant was determined by a summation of the individual analyses of leaves and stem. An increase in the total boron content of the plants can be traced through the first four or five sampling periods; thereafter the total boron content appeared to be constant. Because of the many separate samples, each of which contained minute amounts of boron, it was not possible to get an accurate estimate of the total.

The total boron content as well as the total weight of the stem increased logarithmically with time (fig 3), but the slope of the total boron curve is less than that of the weight curve, indicating a continued but diminishing source of boron for this organ. Also plotted on figure 3 is the change in boron concentration (ppm) of the stem with time. Since concentration is a function of weight and total boron, it must also change logarithmically with time with a negative slope.

The change in boron concentration with time for even numbered leaves (6–20 inclusive) is given in figure 4. The slopes of the regression lines (b values) and the corresponding correlation coefficients (r values) for all leaves through 18 are given in table I. The boron concentration decreases with time during the periods 1 through 7 for all leaves. This decrease in concentration follows a close linear relationship for the leaves 8 through 15 as indicated by r values numerically greater than −0.8. Older and younger leaves have a less precise relation between the change in concentration and time. In the older leaves, the numerically smaller b values indicate less decrease in boron concentration and the r values indicate a less reliable estimate of the effect. In the younger leaves the slope was steep, indicating a rapid change in boron concentration, but the number of dates over which the regression was calculated was necessarily decreased; therefore, the estimate of the effect was less precise.

Because deficiency symptoms occurred at the seventh harvest period, it was expected that the boron concentration would reach a minimum at the seventh period and remain relatively constant after this date. To test this hypothesis, linear regression was calculated for periods 7 through 12. The r and b values are given in table I. Except for leaf 6 all values are negative, indicating a decrease in boron concentration. Leaf 6 did not have sufficient number of values to accurately determine the trend of the change in boron concentration, although the r value is statistically significant ($P = 0.05$). Most of the r values lack statistical significance and the b values are numerically much less than those for the period 1 through 7, indicating only slight decrease in boron concentration during the period.

The effects described above are illustrated by the broken lines and "x" data points in figure 4. Only

TABLE I

CORRELATION COEFFICIENTS (r) & SLOPES OF LINEAR REGRESSION (b) FOR CHANGES IN TOTAL BORON & BORON CONCENTRATION WITH TIME IN BROCCOLI LEAVES

LEAF NO. (FROM BASE OF PLANT)	Ppm BORON IN LEAF				MICROGRAMS BORON PER LEAF			
	PERIODS 1 THROUGH 7		PERIODS 7 THROUGH 12		PERIODS 1 THROUGH 7		PERIODS 7 THROUGH 12	
	r	b	r	b	r	b	r	b
1, 2, 3	−0.581 **	−7.65	*		−0.134 NS	−0.07	*	
4	−0.505 **	−5.02	*		0.00 NS	+0.02	*	
5	−0.477 **	−3.81	*		−0.106 NS	−0.24	*	
6	−0.570 ***	−4.62	+0.563 **	+4.76	−0.032 NS	−0.125	0.0 NS	0.0
7	−0.257 NS	−4.20	−0.263 NS	−1.32	−0.144 NS	−0.50	+0.118 NS	+0.38
8	−0.832 ***	−5.67	−0.122 NS	−0.53	−0.611 ***	−2.25	−0.254 NS	−0.786
9	−0.849 ***	−5.49	−0.332 NS	−1.21	−0.677 ***	−2.46	−0.319 NS	−0.957
10	−0.882 ***	−6.63	−0.177 NS	−0.34	−0.726 ***	−2.88	−0.125 NS	−0.27
11	−0.909 ***	−6.69	−0.175 NS	−0.30	−0.737 ***	−2.45	−0.233 NS	−0.50
12	−0.940 ***	−6.94	−0.387 NS	−0.56	−0.633 ***	−2.00	−0.273 NS	−0.58
13	−0.895 ***	−6.25	−0.367 NS	−0.37				
14	−0.857 ***	−6.32	−0.466 **	−0.49				
15	−0.804 ***	−6.91	−0.341 NS	−0.43				
16	−0.734 ***	−6.70	−0.435 **	−0.70				
17	−0.571 **	−6.82	−0.510 **	−1.01				
18	−0.488 NS	−7.44	−0.649 ***	−1.97				

* Leaves dead following the omission of boron from the nutrient solution
** Significant at $P = 0.05$
*** Significant at $P = 0.01$

TABLE II

PARTS PER MILLION BORON IN BROCCOLI LEAVES* DURING LAST SIX 4-DAY INTERVALS WITHOUT BORON

LEAF** NO.	HARVEST INTERVAL						
	6	7	8	9	10	11	12
22	33.5	29.0	14.8	4.8	4.0	2.2	2.0
24			16.3	6.7	5.5	2.5	2.0
26			24.0	11.0	9.3	3.2	1.8
28				13.0	23.5	4.2	2.2
30						5.0	5.2

* Mean of four plants
** Leaves numbered from base of plant

calculated regression lines are shown. Data points or lines are omitted when they make the figure difficult to read.

The change in total boron content of leaves 4 to 12 inclusive during the no-boron growing period is illustrated in table I. For leaves 4 to 7, inclusive, the correlation coefficients were not statistically significant and the slope of regression line for the periods 1 to 7 inclusive was nearly horizontal. These indexes indicate that there was little or no net movement of boron into or out of these leaves during this period. However, leaves 8 to 12 inclusive have significant correlation coefficients and steep negative linear regression during the periods 1 to 7 inclusive, indicating positively that boron was withdrawn from these leaves at a relatively constant rate, presumably for use elsewhere in the growing plant. For the period 7 to 12 inclusive there was little or no net movement of boron from any of the above leaves as indicated by the nonsignificant correlation coefficients and the slight slopes of the regression.

The change in total boron content with time for the even numbered leaves 6 through 20 is shown in figure 4 by the dotted line, or by the triangular data points. Since the leaves were also changing in total weight, it is helpful to observe the change in total boron content in relation to the change in weight of the leaf. The solid line and circular data points in figure 4 are the dry weight of the leaf. The line shown is a section of a parabola fitted by the method of least squares. In the youngest leaves the line proceeds off the figure; the portion not shown is not necessary to interpret the boron movement in the plant.

For leaves 13 to 18 inclusive, the change in total boron content with time is complex. Leaf 16 illustrates these changes effectively. Since this leaf was not yet formed at periods 1 or 2, the first samples were obtained on period 3. The weight increased rapidly (*solid line*) with a corresponding increase in total boron (*triangles*) through the fifth period. The weight continued to increase along a sharply rising parabolic curve, but the total boron content decreased to a minimum value at the seventh and eighth periods after which it remained approximately constant. Leaves 13 to 20 inclusive follow a similar trend but with different starting dates.

The concentration curves (ppm) (*broken lines* or *"x" data points* in fig 4) show significant regression with negative slope (table I) through period 7. Only in leaves 17 and 18 does there appear to be any deviation from the linear regression of concentration (ppm) on time. In these two leaves there was an increase from the first to the second date the leaves were sampled (periods 4–5) before the concentration decreased. In the youngest leaves the concentration continues to decline after symptoms have appeared (periods 7–12).

Leaves younger than 20 were also analyzed. Most of these were formed after deficiency symptoms appeared elsewhere on the plant. The concentration of boron (ppm) for leaves 22, 24, 26, 28, and 30 is given

TABLE III

MICROGRAMS BORON PER LEAF IN BROCCOLI* DURING LAST SIX 4-DAY INTERVALS WITHOUT BORON

LEAF** NO.	HARVEST INTERVAL						
	6	7	8	9	10	11	12
22	18.0	18.0	16.5	9.8	8.5	4.0	8.0
24			12.7	15.0	10.0	4.5	11.0
26			6.0	9.0	9.3	4.5	6.8
28				13.0	12.0	6.0	8.5
30						5.2	11.0

* Mean of four plants
** Leaves numbered from base of plant

in table II. There is a definite trend in all leaves except 30 for the concentration to decrease as the leaf continued to grow. The older leaf (no. 22) showed a pronounced tendency to decrease in total content with time, but this tendency was not definite in the leaves formed after the plant showed deficiency symptoms (table III).

Discussion & Conclusions

Broccoli was grown to about a mid-point in its lifetime span with an adequate supply of boron (nutrient solution contained 0.5 ppm B). This resulted in a tissue content of about 50 ppm B. At this time the plants were supplied with a nutrient containing no boron (actually the plant tops continued to increase slightly in total boron content for the next 16 days). From the time the plants received no boron to 44 days later, the growth of the total plant top and each of its leaves was observed and the boron content of each leaf and the stem was determined. When expressed as micrograms per leaf (or stem) it is apparent that there is a significant net movement of the boron within the plant to newer or younger tissues. Not all of the leaves and the stem participate to the same extent in boron re-distribution. The stem continued to increase in weight and continued to accumulate boron for the entire 44-day period. The lower leaves, no. 5 and older, did not show any loss of boron. Leaf 6 showed a decrease in boron concentration but no net movement of boron from the leaf. Leaves 7 to 22 inclusive showed a decrease in total boron with time during a part of the period that the plant was grown without an external boron supply. The older leaves started losing boron immediately upon entering the growth period without external boron, while younger leaves accumulated boron even though none was available from the nutrient solution. This accumulation is evident in leaves 12 to 22 inclusive. After accumulating boron for 16 days or less (while the leaves were young) these leaves began to lose boron. The date of maximum boron accumulation tended to occur at a later date in the younger leaves. After boron deficiency symptoms appeared, none of the leaves accumulated significant amounts of boron.

Re-use of boron is indicated by the continual movement of boron to younger tissues after the external source was removed. The decrease in total boron content of most leaves indicates the source of the boron for each new leaf formed. As long as boron was available for transport, the newly formed leaves would have a high boron concentration, 16 ppm or greater (table II). When mobile boron was nearing exhaustion as in the 11 and 12 harvest periods, the newest formed leaves (no. 29–35) had an average concentration of 5.7 ppm, which is not greatly different from somewhat older leaves on that same date (table II).

There is a practical implication of boron movement in sampling broccoli for analysis to determine the adequacy of fertility. The youngest leaves will maintain a moderately high boron concentration because the internal boron will constantly move in this direction. These leaves will not be best for sampling. The oldest leaves do not release boron for re-distribution. These will not be useful for sampling. Young, but well-formed leaves such as 12 or 14 in figure 4 show rapid reduction in boron concentration down to values of 7 ppm or less when deficiency symptoms appeared. Such leaves will make ideal tissues for leaf analysis.

Summary

Broccoli was grown on a complete nutrient solution with 0.5 ppm B. At about 45 days before flowering the nutrient solution was changed to boronless nutrient. On this date and for the next 44 days at 4-day intervals, four plants were harvested, weighed, and the leaves stripped from the stem. Each leaf was dried and analyzed separately for boron. At 24 days, boron deficiency symptoms developed. As growth continued while the plants were without an external source of boron, a re-distribution of the internal boron occurred. It was continually moved to the youngest tissues. There was no loss from the basal leaves, but marked changes occurred in the younger leaves. For example, the 22nd leaf from the base changed in concentration from 33 to 2 ppm and from 18 to about six micrograms.

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ISOLATION OF METABOLICALLY-ACTIVE SUBCELLULAR PARTICLES FROM ETIOLATED COTTON SEEDLING HYPOCOTYLS USING BOVINE SERUM ALBUMIN IN PREPARATIVE MEDIUM [1, 2]

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Subcellular particles capable of oxidizing various members of the tricarboxylic acid cycle have been isolated from a number of plant sources, as evidenced by the numerous references in various reviews (6, 8, 9, 11). As far as the author is aware, there have been no reports of such particulate preparations being obtained from the cotton plant. In view of the demonstration of particulate activity from a rather wide range of other plant species and tissues, it was assumed that similar preparations could be obtained from cotton plants. A metabolically-active fraction from this source was desired to use in a study of some physiological aspects of Verticillium wilt. The present report accounts some of the difficulties that were encountered in the isolation attempts and lists the results that were obtained.

MATERIALS & METHODS

SOURCE OF TISSUE: Cotton seeds (*Gossypium hirsutum* L. var. Acala 1517C) were surface sterilized with 0.5 % sodium hypochlorite and rinsed thoroughly with tap water and then distilled water. They were then planted in trays of pasteurized sand in the dark. After 5 to 7 days, when the seedlings were about five to seven inches tall, hypocotyls were harvested and cut into segments 1 to 1.5 cm in length and chilled before they were used. When soybean (*Glycine max* (L.) Merr. var. Dorman) hypocotyls were used, the seedlings were grown and treated in the same manner.

PREPARATION OF PARTICULATE FRACTIONS: The preparative procedure followed that of Bonner and Millerd (3), Millerd (14) and Switzer and Smith (17), except when tissue was disrupted by blending. The extraction medium was basically 0.4 M sucrose-0.1 M Na_2HPO_4-KH_2PO_4 at pH 7.0. These concentrations and pH were held constant when other materials were included. The hypocotyl segments, in 25 to 50 gram lots, were processed in the following volumes of medium per gram tissue: 1 ml for mortar grinding or 5 ml for blending, 0.8 to 1.6 ml for washing the pellets, and 0.2 ml for final suspension. Blending time was 30 seconds at line voltage. A loose fitting power-driven glass homogenizer was used to resuspend the pellets. All operations were conducted at temperatures as near freezing as possible. Final suspension was always in the basic sucrose-phosphate medium.

TECHNIQUE OF MEASUREMENT: Oxygen consumption was measured at 30° C using standard Warburg technique (19). As a general rule, 0.7 ml particulate preparation containing 0.2 to 0.5 mg nitrogen was used in each flask. Total nitrogen was determined by the micro-Kjeldahl method, distilling into 4 % boric acid. The reaction mixture contained sucrose, phosphate (pH 7.0), $MgSO_4$, ATP, and cytochrome c. Substrate, glutamate, and DPN were added where indicated. The total volume in each flask was 3.0 ml, including 0.2 ml 20 % KOH in the center well. All reaction components were placed in the main compartment. The particulate preparation was added just before the flasks were placed on the manometers and the entire system was equilibrated to temperature for 10 minutes. Readings were made at 10 minute intervals for 70 minutes. Oxygen consumption rates, with air as the gas phase, were calculated for the 20 to 50 minute interval and are reported on a nitrogen basis. In instances where rates were not linear the 20 to 50 minute period was usually the most stable portion of the curve. All rates reported are net for substrate oxidation corrected for endogenous respiration. In almost all instances, duplicate flasks were used for each determination and the averages are reported.

Cytochrome c, ATP (99 %+, Disodium, Chromatographed), DPN (Chromotographically Pure), and BSA (Fraction V Powder) were obtained from Nutritional Biochemicals Corp.

RESULTS & DISCUSSION

FRACTIONS PREPARED WITH SUCROSE-PHOSPHATE & ADDITIVES OTHER THAN BSA: The data in table I show that somewhat standard techniques used for other plant material were not adequate for isolating an active succinoxidase particulate preparation form etiolated cotton seedlings. Repeated efforts using a sucrose-phosphate medium, used for extraction of

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mung bean (3, 14) and soybean (17) particles, resulted in fractions from cotton hypocotyls showing no more than negligible succinoxidase activity. This was true with either mortar or blender preparations. Additional trials, data not reported, showed that sucrose alone, variations in sucrose and phosphate concentrations, variations in pH, and alteration of centrifugal forces during centrifugation had no advantageous effects. Various additives included in the extraction medium and sometimes in the washing medium were of little value in enhancing the succinoxidase activity of the preparations. Cysteine and reduced glutathione (7, 16) and EDTA[3] (13, 15, 18), reported to enhance the activity of some preparations, caused minor increases in activity. PVP, also suggested as having some advantageous effect (20), did not essentially improve the preparations. Tris buffer, succinate, gelatin, and egg albumin were of equally small value or less.

The data in table II help to explain why the above mentioned preparations contained very low or no succinoxidase activity. When cotton and soybean fractions were prepared identically, according to Switzer and Smith (17), the soybean preparations generally exhibited $Qo_2(N)$'s in the range of 400 to 600 while the cotton preparations were inactive or very nearly so. Soybean succinoxidase activity decreased very rapidly when aliquots of the cotton fractions were added to flasks containing the soybean system. As shown in table II, inhibition was still very pronounced even when the amount of added cotton preparation was reduced to less than half.

[3] Abbreviations used in text: EDTA (ethylene diamine tetra-acetic acid, disodium salt), PVP (polyvinylpyrrolidone), BSA (bovine serum albumin), tris (trishydroxmethylamino methane), ATP (adenosine triphosphate), DPN (diphosphopyridine nucleotide).

TABLE I

EFFECT OF VARIOUS ADDITIVES IN GRINDING & WASHING MEDIUM ON SUCCINOXIDASE ACTIVITY OF ETIOLATED COTTON SEEDLING HYPOCOTYL PARTICULATE FRACTIONS*

ADDITIVE IN GRINDING MEDIUM**	ADDITIVE CONC	METHOD OF PREPARATION	ADDITIVE IN WASH MEDIUM**	$Qo_2(N)$
None		Mortar	None	16
		Blender	None	10
Cysteine	0.005–0.1 M***	Mortar	None	21–40
	0.04 & 0.1 M	Mortar	Cysteine	21, 40
	0.16 & 0.3 M	Blender	None	53, 55
	0.3 M	Blender	Cysteine	36
EDTA	0.1 M	Mortar	None	22
		Mortar	EDTA	18
		Blender	None	62
	0.05 M	Blender	None	0, 0
	0.1 M	Mortar	No wash	0
Tris	0.1 M	Mortar	None	25
		Mortar	No wash	0
		Blender	None	0, 0
PVP	5 % (w/v)	Blender	None	19
	10 %	Mortar	None	31
		Mortar	PVP	17
Reduced glutathione	0.004–0.12 M***	Mortar	None	36–55
	0.031 M	Blender	None	70
Succinate	0.1 M	Mortar	None	23, 34
		Blender	None	0
		Mortar	Succinate	35
Gelatin	0.5 % (w/v)	Blender	None	13
	1 %	Mortar	None	0
Egg Albumin	2 % (w/v)	Blender	Egg alb.	38

* Reaction mixture contained 0.2 M sucrose, 0.05 M phosphate (pH 7.0), 10^{-3} M $MgSO_4$, 5×10^{-4} M ATP, 3.25×10^{-5} M cytochrome c, and 0.02 M succinate.

** Grinding and washing medium was 0.4 M sucrose-0.1 M phosphate, pH 7.0. Additives included in this medium at concentrations indicated.

*** Indicates range of concentrations tried.

TABLE II

EFFECT OF ADDING PORTIONS OF COTTON FRACTIONS TO A SOYBEAN SUCCINOXIDASE SYSTEM*,**

FRACTIONS USED***	SOYBEAN-COTTON RATIO IN FLASKS	% INHIBITION OR STIMULATION
SB + C	1-1	−55 to −99 % (Avg = −77 %)
	1-0.71	−62, −92 %
	1-0.43	−40, −80, −82 %
	1-0.14	−2 %
SB + C-S	1-1	−26, −20, +47 %
SB + C-B	1-1	−29, −43, −61 %
SB + C-B-S	1-1	+11 to +46 % (Avg = +27 %)
	1-0.71	+12 %
	1-0.43	+6 %
SB +C-B-P	1-1	+2 to −44 % (Avg = −19 %)
	1-0.71	−15, −25 %
	1-0.43	−5, −15 %
	1-0.14	−2 %
SB + C (Tissue mixed)	1-1	−68 %
SB + C (Extracts mixed)	1-1	−89 %

* Soybean and cotton fractions prepared identically by grinding in a mortar with sucrose-phosphate, followed by one washing and finally suspending in same medium.

** Reaction mixture same as in table I.

*** Abbreviations used in table: SB, soybean particulate fraction; C, cotton particulate fraction; C-S, supernatant from first high speed spin of cotton extract; C-B, boiled (10 min) cotton particulate fraction; C-B-S, supernatant from boiled cotton particulate fraction after centrifugation; C-B-P, resuspended sediment from boiled cotton particulate fraction after centrifugation.

The cotton fractions undoubtedly contained a toxic material or materials that acted on the soybean system when the two were mixed. It seems reasonable that the enzyme systems of the cotton fractions were similarly inhibited during the preparative procedure, explaining why little or no succinoxidase activity could be measured. Cotton seedlings apparently contain the inhibiting substance in an inactive form or

TABLE III

SUCCINOXIDASE ACTIVITY OF COTTON PARTICULATE PREPARATIONS WHEN BSA WAS INCLUDED IN EXTRACTION MEDIUM*,**

EXPERIMENT NO.	METHOD OF PREPARATION	$Qo_2(N)$ AT BSA CONC (W/V)				
		0.5 %	1 %	2 %	3 %	5 %
5-4	Blender			167		
5-6	"		119			
5-16	"			142	198	
	Mortar				75	
5-11	Blender		147			
5-20	"		204	232		
5-23	"	188			301	
5-25	"					221
5-30	"	85	128	171	187	228
5-31	Mortar	18	30	48	63	72
5-10	Blender			167		
	" (1-3 Tissue-volume ratio)			98		
6-15***	Blender		303	534		

* Fractions washed once with sucrose-phosphate and suspended in same medium.

** Reaction mixtures same as in table I.

*** Fraction washed once with BSA solution, then with sucrose-phosphate alone, suspended in same medium, 0.043 M glutamate present in reaction mixture.

TABLE IV

EFFECT OF ADDING EQUAL PORTIONS OF BSA-PREPARED COTTON FRACTIONS TO SOYBEAN SUCCINOXIDASE SYSTEM*

FRACTIONS USED**	METHOD OF COTTON PREPARATION	BSA CONC (W/V)	EFFECT ON SOYBEAN SYSTEM
SB+C	Blender	1 %	−32, −57 %
SB+C	Blender	2 %	−14, −50 %
	Blender	2 % (Blend & wash)	−30 %
SB+C-S	Blender	2 % (Blend & wash)	+22 %
SB+C	Blender	3 %	−30 %
	Mortar	3 %	−54 %

* Soybean fractions prepared as in table II, cotton fractions prepared as in table III, reaction mixtures same as in table I.
** Abbreviations same as in table II.

confined to vacuoles and as such it does not affect the cytoplasmic particles. Upon cell disruption, however, the material is released and exerts its toxic effect. Some of the material becomes rather tightly bound to the particles during isolation since washed particle preparations retained their toxic activity.

Further data in table II shows that aliquots of the supernatant from the cotton extraction process were variable in their toxic action and that boiling the various fractions reduced but did not eliminate the inhibitory effect. Further work is needed to characterize the toxic principle.

FRACTIONS PREPARED WITH SUCROSE-PHOSPHATE PLUS BSA: The report of Price and Thimann (15) that inert proteins, particularly BSA, had some stabilizing and enhancing effect on particulate preparations led to the inclusion of BSA in the present work. Their work with pea stems showed that this material exerted some effect if added to the washing medium and to the reaction mixture but had no effect in the extraction medium. In the present work, it was used in the extraction process but not in the reaction mixtures.

The data in table III show that considerably higher succinoxidase activity was found in cotton preparations when BSA was added to the grinding medium than was found previously with other additives. Endogenous respiration ranged from none in most cases to less than 5 % of substrate-induced respiration. In general, higher BSA concentration led to more active preparations. This is particularly evident in the results from experiments 5-30 and 5-31 where direct comparisons were made between BSA-concentration effects. These two experiments also show that blender preparations were superior to mortar preparations. This was probably due in part to the greater amount of BSA per unit of cotton tissue in the blender method. Where a blender was used, a 1:3 tissue:volume ratio was inferior to a 1:5 tissue:volume ratio at the same BSA concentration. However, a comparison between the 5 % BSA mortar preparation and the 1 % BSA blender preparation shows that the mortar method produced a less active

TABLE V

EFFECT OF INCLUDING 2 % BSA IN WASHING MEDIUM ON SUCCINOXIDASE ACTIVITY OF FRACTIONS PREPARED BY BLENDING IN SUCROSE-PHOSPHATE-2 % BSA*, **

TREATMENT	Qo_2 (N)
Washed once with S–P (sucrose-phosphate)	120
" twice " "	96
" once with BSA, once with S–P	199
" twice " " , " " "	211
Washed once with S–P	297
" twice " "	292
" once with BSA, once with S–P	639
" twice " " , " " "	662
Washed once with S–P	167
" " " BSA, once with S–P	256
Washed once with S–P (1–3 tissue volume ratio)	98
" " " BSA, once with S–P	105

* Reaction mixtures same as in table I.
** 2 % BSA on w/v basis.

preparation, though the two methods had the same ratio of tissue to total BSA present. It is possible that the violent blender action led to a more rapid association between the BSA and the toxic material. The mechanism whereby BSA exerts its protective action is unknown but it is assumed that it combines in some way with the toxic material released in the grinding process. Thus, the inhibiting substance is not free to act on the particles. It is clear that BSA exerts much more than a stabilizing effect on cotton preparations. Higher BSA concentrations were required, however, than those used by Price and Thimann (15) for pea particles.

Further evidence that BSA suppressed the inhibitory action is shown in table IV. Aliquots from cotton fractions prepared with BSA were less toxic to the soybean succinoxidase system than were similar aliquots from non-BSA preparations, already noted in table II. The inhibitory effect was not completely removed when BSA was used but it was considerably reduced. This coincides with the higher succinoxidase activity of cotton fractions themselves when prepared with BSA, as already noted.

The data in table V show that the protective action of BSA is increased when included in the washing medium as well. After blending in 2% BSA (in sucrose-phosphate), a further wash of the particles with this same medium, before a final wash with sucrose-phosphate, essentially doubled the succinoxidase activity of that where only sucrose-phosphate

TABLE VI

EFFECT OF VARIOUS COFACTORS & CONDITIONS ON SUCCINOXIDASE ACTIVITY OF COTTON PARTICULATE FRACTIONS PREPARED BY BLENDING & WASHING WITH SUCROSE-PHOSPHATE-2% BSA

TREATMENT	Qo_2 (N)	% COMPLETE SYSTEM*, **
Without magnesium	680	95
Without cytochrome c	394	70
	402	71
	468	75
	403	88
	345	68
1.62×10^{-5} M cytochrome c	472	103
6.50×10^{-5} M cytochrome c	509	111
3.25×10^{-5} M cytochrome c	754	99
Without cytochrome c, without glutamate	229	37
	132	25
Without glutamate	400	56
	384	50
	286	46
	154	29
	163	31
Without ATP, without glutamate	325	45
	150	20
	109	21
Without ATP	667	93
	691	95
2.5×10^{-4} M ATP	720	99
1.0×10^{-3} M ATP	711	98
With DPN (1 mg/flask)	432	89
pH 6.0	376	50
pH 8.0	511	67
10^{-2} M Malonate	41	7
10^{-3} M "	269	46
10^{-4} M Cyanide	80	14
10^{-5} M "	317	54
10^{-6} M "	604	103

* Complete system contained 0.2 M sucrose, 0.05 M phosphate (pH 7.0), 5×10^{-4} M ATP, 3.25×10^{-5} M cytochrome c, 0.043 M glutamate, and 0.2 M succinate.

** A control containing the complete system was run for each preparation and experiment. Endogenous respiration in these preparations was less than 5% of substrate-induced respiration.

was used to wash the particles. A second BSA wash resulted in minor increases in activity. This decided stimulation of activity by including BSA in the washing medium agrees in substance with the report of Price and Thimann (15). In the one instance tried, a BSA wash did not show the decided stimulation when the blending volume was reduced to a 1:3 rather than 1:5 ratio.

Table VI shows some characteristics of the succinoxidase system from etiolated cotton seedling hypocotyls prepared by blending 30 seconds with sucrose-phosphate-2 % BSA, washing once with the same medium, washing again with sucrose-phosphate, and final suspension in sucrose-phosphate. These particle preparations were similar in most respects to other plant preparations, including the rate of utilization of succinate (1, 2, 14, 15, 17). Excluding magnesium from the reaction mixture had perhaps a slight depressing effect but not to the extent reported for mung beans (14) and soybeans (17). The system responded to added cytochrome c, this material causing about 30 % stimulation. There have been reports of cytochrome c stimulation (10, 12, 17) and non-stimulation (2, 4, 18) of plant preparations. The effect of cytochrome c was about the same with or without glutamate in the reaction mixture. Glutamate addition to the reaction mixture caused the greatest response of any of the factors tested. This stimulation by glutamate was probably due to the trapping of oxalacetate by transaminase activity, thus preventing the accumulation of oxalacetate with its inhibitory activity (10). Without glutamate the rates were not only slower but were generally not linear for the 70 minute measurement period. The effect of omitting ATP was

TABLE VII

OXIDATION OF VARIOUS SUBSTRATES BY COTTON PARTICULATE FRACTIONS PREPARED BY BLENDING & WASHING WITH SUCROSE-PHOSPHATE-2 % BSA*

SUBSTRATE	$Qo_2(N)$		SUCCINOXIDASE $Qo_2(N)$**
	WITHOUT DPN	WITH DPN	
Citrate (0.02 M)	20	15	304
	4	0	591
	55(30)***		
Fumarate (0.02 M)	31	123	479
	69	157	591
		79(59)	
Isocitrate (0.02 M)	20	49	304
	7	75	533
		62(101)	
Alpha-ketoglutarate (0.02 M)	94	62	479
	58	52	484
	73(30)		
Malate (0.02 M)	24	110	479
	35	112	484
		89(50)	
"Sparker" malate (0.0017 M)	7	16	304
	42	31	591
	(17)		
Pyruvate (0.02 M) + "Sparker" malate (0.0017 M)	35	44	304
	69	75	591
	(37)		
Pyruvate (0.02 M)	23	20	304
	38	46	591
	48(0)		
Oxalacetate (0.02 M)	0	0	304
	19	0	591
	4(0)		
Glutamate (0.043 M)		16	260
	31	61	533
	18	48	304
	37	87	479
	21	87	591
	0	48	484

* Reaction mixtures same as in table VI; DPN added at 1 mg/flask when shown.
** Succinoxidase activity of same preparations.
*** Figures in parentheses are rates without glutamate in the reaction mixtures; where these coincide with figures not in parentheses, they are direct comparisons on the same preparation.

very slight, agreeing with the report of Biale et al (2) for avocado fruit, Price and Thimann (15) for peas, and Millerd (14) for mung beans, but not the reports of Switzer and Smith (17) for soybeans, and Davies (4) for peas. The presence of DPN had no stimulating effect on succinoxidase activity and even had a small depressing effect. This latter effect was mentioned by Beevers and Walker (1). The lack of response to DPN is to be expected from the general knowledge of a succinoxidase system. Rates were considerably less at pH 6.0 and 8.0, indicating a pH optimum somewhere near neutrality as has been reported for other preparations (12, 14, 17, 18). The system was sensitive to both malonate and cyanide, as expected from the nature of these two inhibitors.

That the particle fraction isolated from cotton seedlings is also capable of oxidizing other substrates is shown in table VII. All the substrates tried were oxidized but at a much slower rate than was succinate, and some were utilized at extremely low rates. Citrate, pyruvate, and oxalacetate comprised this latter group. No evidence was found for increased pyruvate oxidation when the reaction mixture contained a low concentration of malate. This is contrary to the reports of Davies (4), Millerd (14), Switzer and Smith (17), and Beevers and Walker (1), among others, for various plant preparations. Of the substrates tested, fumarate, isocitrate, and malate responded to added DPN, as did glutamate. There was no response of alpha-ketoglutarate to DPN, as some reports have indicated (1, 15, 17). The utilization of glutamate, though slow, agrees with the reports of Switzer and Smith (17) and Freebairn and Remmert (5). Omitting glutamate from the reaction apparently caused some decrease in oxidation rates of the various substrates tested except for isocitrate, in which case there was possibly some increase. The low rates for oxalacetate prohibit drawing conclusions as to the glutamate effect.

The rather slow utilization of the various substrates tested, except for succinate, may be due to the lack of a full complement of necessary cofactors in the present work. It is also possible that even with BSA in the preparative medium the resulting particle fractions were not completely free of the inhibiting material and as such were isolated under sub-optimum conditions.

Summary

Difficulties encountered in attempting to isolate enzymatically-active subcellular particles from etiolated cotton seedling hypocotyls were found to be largely associated with the presence of a toxic substance or substances in the seedlings. This toxic principle apparently inactivated the particles during the isolation procedure and was active against soybean succinoxidase activity.

Inclusion of bovine serum albumin at rather high concentrations in the preparative medium facilitated the isolation of active particles. This material apparently neutralized the inhibiting substance in some manner. Other materials tried and found to be of no value in securing active preparations were cysteine, ethylenediamine tetra-acetic acid, polyvinylpyrrolidone, reduced glutathione, succinate, gelatin, and egg albumin.

Fractions prepared by blending the hypocotyl segments were superior to those prepared by grinding in a mortar.

The resulting particle preparations were similar to other plant preparations with respect to cofactor requirements. Glutamate added to the reaction mixtures markedly increased the rate and stability of oxidation of succinate but not the other substrates. Succinate was oxidized very readily, while citrate, pyruvate, and oxalacetate were oxidized very slowly. Other substrates utilized fairly readily were fumarate, isocitrate, alpha-ketoglutarate, malate, and glutamate. Oxidation of fumarate, isocitrate, malate, and glutamate was stimulated by added diphosphopyridine nucleotide.

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TWO IMPROVED & INEXPENSIVE SYSTEMS FOR MOISTURE STABILIZATION IN SEEDS OR OTHER TISSUES [1, 2]

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Devices to control relative humidity have long been used by plant scientists. Some control systems are rather complex ones with humidistats, radio tubes, pumps, and fans (8). Alterations of seed moisture have been effected by such techniques as pouring known amounts of water over known amounts of seeds, achieving equilibrium at low temperatures in stoppered jars within a few days (2).

Perhaps the most commonly used method to vary seed moisture is storage in closed containers, over salts or solutions of known vapor pressures, for a few days or weeks (e.g., 7). There are several objections to this technique:

I. There is a vapor pressure gradient from the salt or solution surface to the other extremity (usually the top) of the chamber.

II. Unless the sample is completely exposed, as from being in a single layer, there is a vapor pressure gradient from interior to exterior of the sample.

III. The atmosphere within the chamber can be greatly altered whenever the container is opened.

IV. It is difficult or impossible to remove the sample without exposing it to a different atmosphere.

V. Ascertaining moisture changes in the sample is difficult without upsetting the system and/or destroying the sample.

VI. Important effects on seed physiology may result from volatile chemicals used for humidity control (4).

Recently, preradiation moisture treatments have been given to seeds in such a way that some of these objections do not apply: seeds were exposed to a constant flow of air that had first gone through a saturated solution calculated to yield the desired relative humidity at the temperature used (3). To improve on this system and render it more flexible, the following two arrangements were devised.

Construction of Systems

Plywood Boxes (see fig 2): Lidless boxes were made of plywood with all joints glued and screwed, and three coats of marine varnish were applied to the interiors. Lids of ¼-inch Lucite were cut and drilled to bolt over rubber gaskets. Open shelves occupying about one-third of the interior volume were devised for use with sample trays of wire screen. With inside dimensions of 17 inches long, 11 inches wide, and 14 inches deep, each box contained about

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[2] This manuscript is published with the permission of the Director of the University of Tennessee Agricultural Experiment Station, Knoxville, Tenn. The work was completed under contract no. AT–40–1–GEN–242 between the University of Tennessee College of Agriculture and the U. S. Atomic Energy Commission.

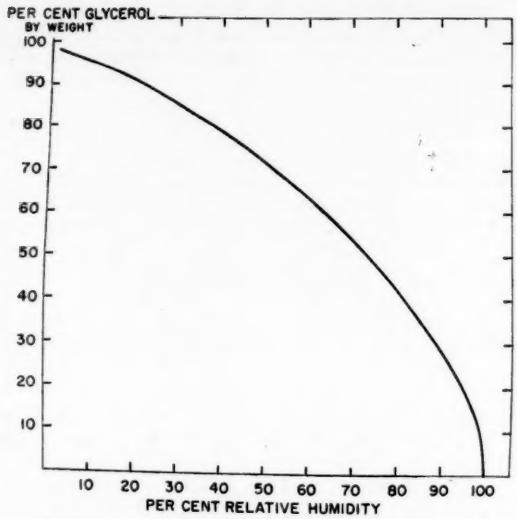


FIG. 1. Influence of per cent glycerol (by weight) in glycerol-water mixtures on relative humidity at 25° C. (Computed from International Critical Tables, Vol. III, p. 291, 1928.)

46 liters. For air circulation, a diaphragm-type (aquarium) pump, displacing about 1 liter/min, was put inside each box; thus the air was changed more than 30 times per day. (A closed pump could be left outside the box.)

For humidity control with a nonvolatile agent, glycerol was used either alone or diluted with water (see fig 1). Tubes piercing the box wall carried air through the conditioning solution (in a flask outside) and back into the box. Bottle and solution were tared at the beginning of an experiment, and the solution replaced when weight had changed significantly. Before dilution, stock glycerol was assayed for water content by Karl Fischer titration with the methods of Carter and Williamson (6, p. 94). Water-free glycerol for 0 % R.H. was obtained by refluxing.

For manipulation without disturbing the enclosed atmosphere, plastic gloves were sealed over ports in adjacent walls (fig 2, C). An airlock for sample removal was made by piercing the wall with a short metal tube closed with rubber stoppers (fig 2, D).

For moisture determination without sample destruction, wire sample trays were replaced with wire cylinders which were weighed, filled with seeds, closed, and reweighed. Simultaneously, moisture was determined in a similar sample by the usual grinding and drying techniques (1, p. 404). A test tube large enough to contain a cylinder of seeds, tared with stopper, was kept in each box. At intervals, a cylinder of seeds was put into the tube (which was then stoppered), removed through the airlock, weighed, and replaced. Knowing sample weight and moisture at the beginning of storage, one could easily compute per cent moisture from the altered weight. Weighing and computing moisture for the nine samples in one box at one time interval took about 15 minutes.

GLASS TUBES: This system retained the airflow and glycerol features of the plywood box system. Improved items were: samples were more easily removed and weighed with less disturbance to the system, a higher sample/air ratio was obtained, and more samples could be treated in a similar bench area.

As depicted by figure 3, this system had each sample in a glass tube an inch or more in diameter and of any practical length, with virtually any number of tubes connected to one humidity regime. The stoppered sample tubes were connected by glass and rubber tubing to galvanized pipe headers, which served as entry and exit chambers for the flow of air. Air was moved by the same diaphragm-type pump, sealed in a wide mouthed jar to maintain a closed system. Air was passed through the proper glycerol solution before reaching the header tube.

Each tube-stopper-clamp unit (ordinarily containing seeds of one species) was tared, half filled with sample, reweighed, and attached to the system. Simultaneously, moisture was determined in a similar sample by the usual grinding and drying techniques (1, p. 404). Thus, moisture could be determined for any sample after any interval simply by weighing and computing, based on the one original determination, without sample destruction. The double arrangement of clamps (fig 3, D) permitted one tube to be removed for weighing without altering either that sample or the remainder of the system.

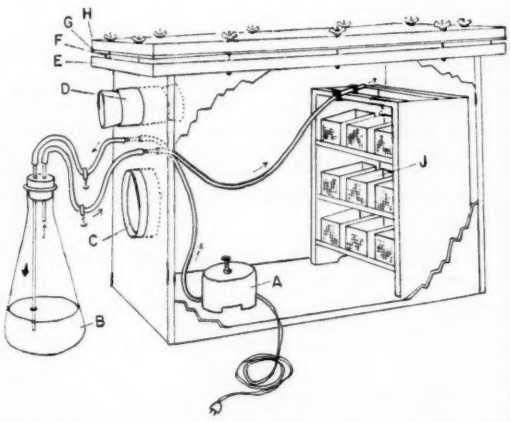


FIG. 2. Plywood box, cutaway view, showing: (A) pump, (B) glycerol flask, (C) manipulation port (plastic glove removed), (D) airlock with rubber stoppers, (E) lip of box, (F) rubber gasket, (G) Lucite top, (H) frame for sealing top, and (J) wire screen sample trays. Side removed in drawing contains another manipulation port. Arrows show direction of air movement.

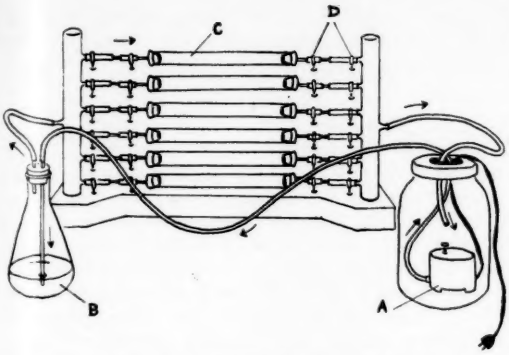


FIG. 3. Glass tube system, showing: (A) pump, (B) glycerol flask, (C) sample tube, and (D) clamps. Arrows show direction of air movement.

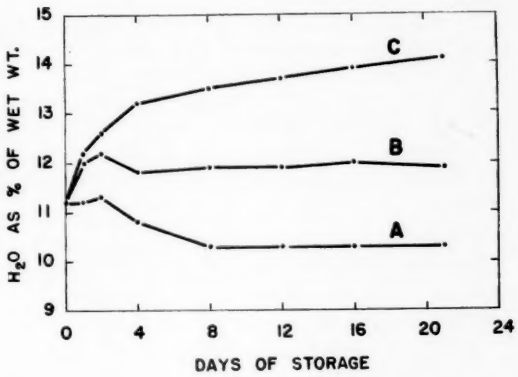
TESTING OF SYSTEMS

PLYWOOD BOXES: After storage for several weeks in a room with relative humidity between 40 and 60 % and temperature between 16 and 24° C, grains of barley (*Hordeum vulgare* L., cv. Holston) were screened to remove extremes of size (5). The grains were then placed in nine wire cylinders in each of three boxes. The kernels weighed approximately 150 g per box. Results of one test series are given in figure 4 and table I.

In this series, the glycerol solutions were deliberately left unchanged during the entire 21 days, to ascertain whether satisfactory spreads of grain moisture could be obtained. Although each box had only 625 ml of glycerol solution to condition 46 liters of air and 150 g of grain, there was a sufficient spread among grains stored at 95 % (fig 4, A), 70 % (fig 4, B), and 40 % glycerol (fig 4, C). These solutions were expected to maintain relative humidities of 12 %, 52 %, and 81 %, respectively.

Variation within boxes was tested by comparing kernel moisture after 21 days' storage for nine positions in three boxes. Results are given in table I; position 1 was lower left, 9 was upper right in each box. There were no significant differences in moisture content due to various positions within the boxes.

GLASS TUBES: Results of a typical series with the glass tubes are depicted in figure 5. In this series, as is ordinarily done, the conditioning liquid was replaced at intervals as necessary. Each humidity group consisted of six sample tubes totaling about



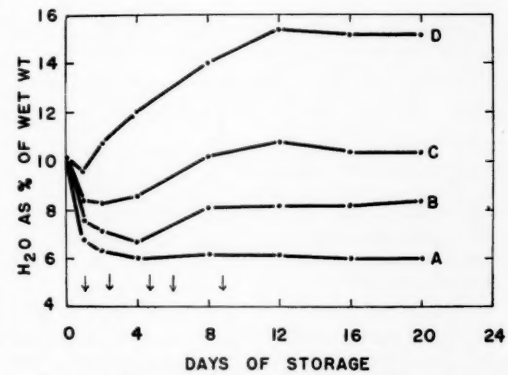


FIG. 4. Moisture changes with time in barley grains stored in plywood boxes at (A) 95 %, (B) 70 %, and (C) 40 % glycerol by weight, corresponding to theoretical relative humidities of (A) 12 %, (B) 52 %, and (C) 81 % at 25° C. Solutions were not replaced during storage.

FIG. 5. Moisture changes with time in wheat grains stored in glass tubes at (A) 100 %, (B) 89 %, (C) 72 %, and (D) 48 % glycerol by weight, corresponding to theoretical relative humidities of (A) 0 %, (B) 25 %, (C) 50 %, and (D) 75 % at 25° C. Arrows show times solutions were replaced.

TABLE I

MOISTURE (AS % OF WET WEIGHT) IN BARLEY GRAINS AFTER 21 DAYS' STORAGE IN PLYWOOD BOXES AT 25° C

Box	% GLYCEROL BY WT	THEORETICAL RELATIVE HUMIDITY (%)	POSITION WITHIN BOX									
			1	2	3	4	5	6	7	8	9	Ave.
A	95	12	10.2	10.5	10.2	10.2	10.2	10.2	10.0	10.6	10.6	10.3
B	70	52	12.1	12.1	12.0	12.1	11.6	11.7	11.8	11.5	12.2	11.9
C	40	81	14.9	14.2	13.5	14.7	14.8	14.5	12.7	13.6	14.0	14.1

Differences within boxes nonsignificant; $F = 1.23$, $DF = 8$, $P = 0.30$.

600 g of seeds and 600 ml of air, conditioned by 600 ml of glycerol solution. An excellent spread of sample moistures was obtained in systems using 100, 89, 72, and 48 % glycerol (0, 25, 50 & 75 % R.H., resp.).

During the first few days, there were small differences in moisture at opposite ends of a tube, but these disappeared after about a week.

Discussion

The two systems (boxes & tubes) merit consideration for control of relative humidity for the following reasons:

I. They are inexpensive: one unit (i.e., one box or one series of tubes) contains readily obtainable materials costing no more than $10, including a $6 aquarium pump.

II. They control vapor pressure with a nonvolatile chemical (glycerol) which should not injure living tissue.

III. They reduce variations of moisture within and between samples by a constant flow of air.

IV. They allow periodic check of moisture changes without destruction of the sample.

V. They permit easy replacement of the conditioning (glycerol) solution.

In addition, the box system permits manipulation within the controlled atmosphere; the tube system allows more rapid measurements with unlimited sample numbers.

The tube system is now used exclusively in this laboratory to precondition seeds at various humidities prior to administering ionizing radiation. For some types of study, where manipulation must be done inside the controlled atmosphere, preference would be given the box system. Doubtless, persons interested in these systems will achieve combinations or modifications to suit their special needs.

Summary

Two inexpensive systems for storing seeds or other tissues at constant relative humidities are described. Air flow is continuous, and vapor pressure is conditioned with a nonvolatile compound (glycerol). Provision is made for easy checking and replacement of the conditioning liquid. Sample moisture is quickly ascertained with little or no interference to the system and without sample destruction. One system (plywood boxes) permits manipulation within the enclosed atmosphere; the other (glass tubes) affords easier sample measurement and virtually limitless sample numbers.

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INVERTASE & INVERTASE INHIBITOR IN POTATO [1]

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The pattern of changes in sugar composition which occur when potatoes are stored at low temperatures or are irradiated (18, 19) suggests that the reducing sugars may arise as the consequence of the hydrolytic action of invertase on sucrose. During cold temperature storage there is a rapid increase in sucrose content followed by an increase in reducing sugars relative to the sucrose content. Furthermore a survey of the literature indicates that, for many varieties of potatoes, the ratio of glucose to fructose is close to unity during at least part of the time of storage (17). If invertase is indeed a factor in the accumulation of reducing sugars, information concerning the characteristics of this enzyme and the inhibitor here reported could conceivably lead to means for controlling the reducing sugar content of potatoes (20).

The literature on potato invertase is rather sparse. In 1903, Kastle and Clark (9) detected invertase in sprouted but not in unsprouted potatoes. In 1928, McGuire and Falk (13) showed that the invertase action of potato juice was higher at pH 4 than at pH 5 and 6. Denny et al (5) found that the invertase activity of potato juice from tubers treated with ethylene chlorohydrin was significantly greater than that from untreated controls. A few reports have suggested that the level of invertase activity depends on the elemental nutrition of the plant [potassium (24), boron (4), chlorine & sulfur (10)]. Bois and Savary (3) and McCready (12) in more recent years have detected invertase in potatoes, although the latter author could find activity only in sprouted and not in unsprouted tubers.

The present paper describes our experience in attempting to detect invertase in crude potato macerates. In general, the results confirm the scattered findings of the older literature but also point to the presence of an endogenous inhibitor of the enzyme. A preliminary report of some of the present results has been published elsewhere (21).

MATERIALS & METHODS

SOURCE OF TUBERS. Four different lots of tubers were used in this investigation. Lot A consisted of freshly harvested (2–3 weeks) tubers of the White Rose variety. Lot B, the same variety, had been stored at 4° C for about one year and had not sprouted. Lot C consisted of tubers from Lot B which were removed from cold storage after 1 year and allowed to remain at room temperature in a humid atmosphere (about 90 % R.H.) for 18 days prior to assay for enzyme activity. Sprouts 1 to 2 inches long had developed. Lot D consisted of Russet Burbank tubers which were assayed about 4 to 6 weeks after harvest.

PREPARATION OF EXTRACT. Five to ten tubers were hand-peeled and diced. Three hundred grams of the dice were mechanically blended with 20 to 30 g of ice and 50 mg of ascorbic acid in a Waring Blendor for 5 minutes. The slurry was passed through cheese cloth and the resultant extract filtered through Celite. The clear filtrate was used for the determination of invertase activity.

INVERTASE DETERMINATION. The invertase reaction mixture contained varying amounts of the above filtrate, buffer, (0.05–0.2 M) water and sucrose (0.065 M final concentration) in a total volume of 7 ml. This concentration of sucrose is quite adequate to saturate the enzyme. Sucrose, when present at a final concentration of 0.040, 0.050, 0.065, 0.080 or 0.140 M, saturated the potato invertase. The same maximum rate of inversion was obtained with each concentration of sucrose. The K_M of crude, dialyzed potato invertase prepared from cold stored potatoes was found to be 0.003 M for sucrose at pH 6.2. The estimated K_M for leaf invertase has been reported as 0.004 M (25). Zero-time controls and controls containing no added sucrose were run, along with the experimental digests at 30° C in the presence of toluene. After the designated time intervals, including a time close to 24 hours, the action of the enzyme was terminated by addition to the reaction mixture, with mixing, of 150 mg of air-dried cation exchange resin [Amberlite IR–120, (H)] plus 2 ml ice water. After 10 minutes at 0° C, 500 mg of anion exchange resin were added [Amberlite IR–4 (OH)]. After 2 hours at 0° C the sample was diluted to 100 ml and filtered through Whatman no. 2 filter paper. This method halts the reaction by creation of a controlled acidic environment (pH 2) at low temperature and precipitates protein in a form which renders the latter easily removable. A satisfactory replication of assays was obtained. The results with the controls demonstrated that enzyme activity was minimal after addition of the

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[2] A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

TABLE I

SUMMARY OF INVERTASE ACTIVITY OF POTATO TUBERS

POTATO LOT*	BUFFER	pH	CONC OF POTATO EXTRACT**	INVERTASE ACTIVITY***
A	None	5.8	1.0	0
B	"	6.2	1.0	3
"	Acetate	4.9	1.0	4
C	Phosphate	4.4	0.13	26
"	"	5.3	0.13	22
"	"	6.3	0.13	17
"	Acetate	3.9	0.13	21
"	"	4.8	0.13	17
"	"	5.8	0.13	16
D	Phosphate	5.7	0.84	3.5
"	"	5.7	0.21	8.7
"	"	5.7	0.09	16.0
"	"	5.0	(0.0)	(26)†

* See text for description of potato lots.
** Expressed as grams of original potato tuber in the reaction mixture.
*** Expressed as mg of sucrose hydrolyzed per 24 hours per g of tuber tissue.
† Calculated from figure 1 and equation I (see text).

ion exchange resins. This ion exchange method eliminated a component of the potato extracts which reacted with copper reagent (used in the sugar analysis) to give rise to a garlic-like odor.

For the determination of sugar, 1 ml of the deionized and diluted enzyme digest was added to 1 ml of a copper-sugar reagent (23). After heating for 20 minutes in a boiling water bath the mixture was cooled and 1 ml of arsenomolybdate color reagent added (14). The optical density of the resulting solution was measured with an Evelyn colorimeter (520 mμ filter). Reducing sugar was estimated from a glucose standardization curve. Invertase activities are expressed as mg of sucrose hydrolyzed per 24 hours per gram of potato tissue.

RESULTS & DISCUSSION

Some typical results are summarized in table I. No invertase under the conditions of the present procedure was detected in freshly harvested White Rose tubers (lot A). An apparently significant invertase activity was detected in this same variety after long term cold storage (lot B). When allowed to remain at room temperature until sprouting started (lot C) considerable activity was present. This activity was about five times that found in the unsprouted tubers.

The results obtained with lot D, consisting of room-stored Russet Burbank potatoes, suggest that the difference between sprouted and unsprouted tubers may not be due entirely to a difference in actual invertase content of the tubers but may reflect the influence of the presence of a naturally occurring inhibitor of invertase activity in non-sprouted tubers. Data obtained with lot D using varying concentrations of potato extract shows that the apparent activity per unit weight of tuber increases with decreasing amount of extract added to the enzyme reaction mixture. Thus, the low values obtained with lot B might have been considerably higher if less than the equivalent of 1 g of tuber per 7 ml of reaction mixture had been used.

Deviation from linearity between enzyme concentration and rate of enzyme action may be interpreted in two ways: A, at higher concentrations of the enzyme the reaction is terminated at the later stage of the reaction where the rate progressively decreases due in part to inhibition by product and in part due to depletion of substrate, so that the overall measured rate per unit of enzyme added appears to be small; B, there is present in the enzyme preparation an inhibitor whose inhibitory effect decreases faster with dilution of the enzyme than does the enzyme activity (8). Situation A is unlikely because the maximum per cent sucrose hydrolyzed in the experiments described here did not amount to more than 7 %. To test the validity of situation B the following equation may be applied:[3]

$$E/V_I = 1/k_1 + cE \qquad I$$

where V_I is the observed enzyme activity in the presence of enzyme concentration E, and k_1 and c are constants. By plotting E/V_I against E, one obtains a straight line if situation B is valid. This straight line will have the intercept $1/k_1$ at E = O, where k is the specific activity in the absence of the inhibitor. A test of the validity of these equations for the invertase activity in crude potato extract is shown in figure 1. It will be noted that the calculated specific activity in the absence of the hypothetical endogenous inhibitor is eight times that in the presence of 0.84 g

of potato tissue in the reaction mixture.

Table II compares data on the magnitude of invertase activity of potato tubers as found in the present studies and in the literature with the invertase activity of other plant sources. On the average, potato tuber invertase activity runs about one order of magnitude lower than that of green leaf tissue. It is vanishingly small as compared with a rich invertase source such as yeast. The metabolic role of invertase in plant tissue, as manifested by its hydrolytic properties, is now a matter of interesting conjecture as the result of investigations of the labeling patterns of sugars obtained from plant organs infiltrated with radioactive sugars (11, 16, 22). Increase in activity upon storage, upon the initiation of sprouting and upon application of sprout inhibitors, as well as the presence of an endogenous inhibitor, suggest the potato tuber to be an excellent system for future studies on the elucidation of the metabolic role of invertase in plant tissue.

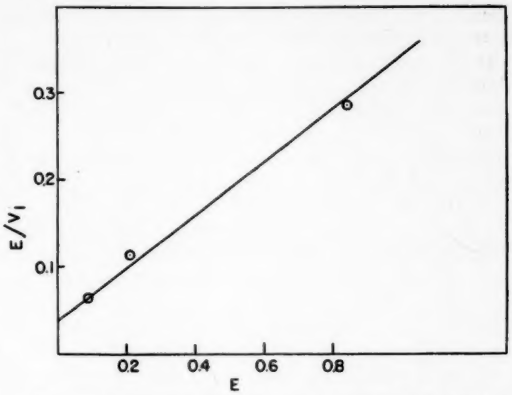

FIG. 1. Demonstration of the presence of an inhibitor of invertase activity in extracts of Russet Burbank potato tubers. E is expressed as g of tuber tissue in the enzyme reaction mixture, and E/V_I as reciprocal of enzyme activity (mg of sucrose hydrolyzed per 24 hr. per g of potato) At E = O, the ordinate is $E/V_0 = 1/k_1$ (see footnote [3]).

[3] The following is a simple derivation for the endogenous inhibition equation: For non-competitive inhibition (7):

$$\frac{V_0}{V_I} = 1 + \frac{I}{K_I} \qquad \text{II}$$

where V_0 = the velocity of enzyme action in the absence of inhibitor, V_I = the velocity in the presence of inhibitor at concentration I, and K_I the equilibrium constant of the inhibitor enzyme complex. Both V_0 and I are proportional to the total amount of enzyme present (E); $V_0 = k_1E$ and $I = k_2E$; k_1 and k_2 are proportionality constants. Substituting these values into equation II:

$$\frac{k_1E}{V_I} = 1 + \frac{k_2E}{K_I} \qquad \text{III}$$

$$\frac{E}{V_I} = \frac{1}{k_1} + cE$$

where $c = \frac{k_2}{k_1K_I}$

When E/V_I is plotted against E, a straight line is obtained and the intercept at E = O is $1/k_1$. The equation for competitive inhibition leads to an equation of the same form.

SUMMARY

The apparent invertase activity of crude extracts of potato tubers has been investigated as a function of post-harvest history, pH, and enzyme concentra-

TABLE II

INVERTASE ACTIVITY OF POTATO TUBERS AS COMPARED TO OTHER PLANT TISSUES

SOURCE	TEMP. 0° C	pH	INVERTASE ACTIVITY*	REFERENCES**
Potato tubers	30	4–6	0–38	Present results
" "	37	4–8	1–8	McGuire (13)
" "	25	6	0–60	McCready (12)
" "	20	...	60	Bois (3)
Potato leaves	38	...	500	Doby (6)
Barley leaves	30	...	320	Archbold (1)
Wheat leaves (seedling)	...	...	750	Ward (25)
Tobacco leaves	25	...	600	Nijholt (15)
Oak leaves	...	6	750	Blagoveschenski (2)
Apple leaves	25	6	300	"
Yeast	25	4.8	34,000	Weidenhagen (26)

* Estimated on the basis of mg of sucrose hydrolyzed 24 hour per gram of fresh tissue.
** Only name of first author given, followed by reference number in Literature Cited.

tion. The invertase activity was found to be about one order of magnitude lower than that recorded in the literature for leaves of potato and other green leaf tissue. The results corroborated previous reports that the apparent activity increases upon sprouting. Preliminary evidence for the presence of an endogenous inhibitor of invertase activity based upon kinetic considerations is presented as well as a simple derivation of the endogenous inhibition equation.

Note. Mention of commercial products throughout this report does not constitute recommendation by the Department of Agriculture.

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MECHANISM OF STIMULATION OF RESPIRATION BY PURINES: I. INHIBITOR STUDIES [1]

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A previous publication (1) reported a striking stimulation of the endogenous respiration of nitrogen-starved Chlorella cells by low concentrations of various purines and purine analogs. This stimulation has since been shown in a large variety of nitrogen-starved microorganisms as well as in some higher plants (2).

Because of the widespread occurrence of this effect and the possibility that a novel regulatory function of intracellular purines might be involved, the mode of action of the purines was further investigated. One means of gaining insight into the possible metabolic pathways which might be involved is by means of metabolic inhibitors with known sites of action. Thus it might be possible to locate the purine effect in reactions involving the cytochrome system, oxidative phosphorylation, amino acid metabolism, or glycolysis, for example, if it could be shown that known specific inhibitors of these systems inhibited the purine effect. This paper discusses the results from such studies and points to the involvement of flavoprotein enzymes and cellular lipids in the respiration which is caused by purines. Further direct evidence for the existence of an electron transport system which is mediated by free purines and is active in the oxidation of storage fat will be presented in future papers (3, 4). (Bach, M. K. Stimulation of oxidation of neutral lipids in Chlorella by purines. Unpublished.)

MATERIALS & METHODS

Chlorella vulgaris Beijerinck (ATCC 11468) cells were cultured and processed as previously described (1). Manometric experiments were conducted at 25° C, in the dark with cells suspended in 0.067 M phosphate buffer, pH 6.0, as described (1).

For preparing C^{14}-labeled cells 500 ml of cell suspension were harvested, washed, and suspended in 0.067 M phosphate buffer pH 6.0 to a final volume of 200 ml. This suspension was distributed between two 500 ml Erlenmeyer flasks each of which had a side arm reservoir and a sampling port. One hundred mg $BaC^{14}O_3$ (2 μc/mg) were weighed into the side arms, magnetic stirrer bars were placed in the main compartments, and the units sealed. Serum stoppers were used to close the sampling tube and the side arm opening. The $C^{14}O_2$ was liberated by slow injection of 2 ml of 30 % $HClO_4$. The flasks were placed side by side on two magnetic stirrers, and a bank of two 20-w fluorescent tubes placed immediately behind them. A second bank of similar lights was suspended directly over the tops of the flasks. An aluminum foil reflector closed the front and a blower circulated room air through the sides to prevent overheating. After 16 hours the contents of the side arm were withdrawn with a hypodermic syringe, and the side arm rinsed twice with water. The unfixed $C^{14}O_2$ was then absorbed by injection of 2 ml 25 % KOH into the side arm. The cells were again washed by centrifugation and suspended in phosphate buffer for use.

The evolution of CO_2 was measured by the direct method of Warburg (13). Total radioactivity was determined by plating the $BaCO_3$ which was recovered from the center wells of the Warburg oxygen flasks. The center wells were rinsed three times into a centrifuge tube with distilled water and the trapped $C^{14}O_2$ precipitated by the addition of a few drops of 12 % $BaCl_2$. The precipitate was washed with methanol and plated as previously described (1). Duplicate flasks were used in all cases. All radioactivities were corrected for self absorption and are expressed for infinite thickness.

In the time course experiments 5 ml cells were suspended with or without 6-mercaptopurine in 25 ml Erlenmeyer flasks and shaken at 25° C in the dark. At given intervals flasks were removed, 3 ml of the contents pipetted into Warburg flasks and the respirometric experiments conducted.

Although results from duplicate flasks in these experiments always agreed to within less than 10 % there was considerable day to day variation (1). As a result, all experiments were conducted at least at two separate occasions and the results thus obtained were averaged after adjustment of the uninhibited controls to the same level (1). Furthermore, experiments were so designed that either all variables were included in the same experiment or, when this was not possible, that at least one of the variables overlapped to afford a comparison of the results. The results must be considered as qualitatively accurate but quantitative differences of 15 to 20 % are of doubtful significance.

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TABLE I

COMPARATIVE EFFECT OF INHIBITORS ON VARIOUS SUBSTRATE RESPIRATIONS IN NITROGEN-STARVED CHLORELLA *

INHIBITOR	ADDITIONS						
	NONE (ENDOGENOUS)	10^{-4} M 6-MERCAPTO PURINE	10^{-4} M 6-CYANO-PURINE	10^{-2} M GLUCOSE	5×10^{-3} M $(NH_4)_2SO_4$	10^{-2} M NA ACET.	5×10^{-3} M NA LINOLEATE
Mercuric chloride, 3×10^{-5} M	0	...	45	91	15	...	†
Cadmium sulfate, 10^{-4} M	−19	73	...	20	0	0	...
Hydrazine hydrate, 10^{-2} M	10	50	...	...	...	0	Progressively to 100
Sodium arsenite, 10^{-3} M **	10	98	90	0	20	41	...
Semicarbazine hydrochloride, 10^{-2} M **	−18	64	...	20	95	...	...
Tryptamine hydrochloride, 5×10^{-3} M **	20	52	...	...	...	0	44
Sodium fluoroacetate, 10^{-4} M ***	0	15	...	71	66	...	...

* Tabular entries are percentage inhibition of respiration. The percentage inhibition of respiration except for endogenous respiration is calculated by the following equation:

$$x = \left[\ 1 - \frac{\frac{S+I}{I} - 1}{\frac{S}{E} - 1}\ \right] \times 100$$

Where S = μl O_2 uptake in the presence of substrate; E = μl O_2 uptake in endogenous respiration; I = μl O_2 uptake in the presence of inhibitor; S + I = μl O_2 uptake in the presence of inhibitor and substrate.

** Cells preincubated with substrate for 45 minutes.

*** Cells preincubated with inhibitor for 60 minutes.

† In the presence of $HgCl_2$ linoleate completely inhibited the respiration of the cells.

RESULTS

The use of inhibitors afforded another approach to the establishment of a clear-cut difference between various substrate respirations and the oxygen uptake caused by the addition of purines to the cells. In table I experiments are summarized in which the response of Chlorella to various inhibitors in the presence of several respiration promoting substrates was compared. The method for calculating the inhibition is based on the assumption that inhibitors do not affect endogenous respiration any differently in the presence of a substrate than in its absence. That this is not always true can be seen from the results obtained with $HgCl_2$ and linoleic acid. While at the concentration used $HgCl_2$ did not inhibit endogenous respiration, a total inhibition of respiration was repeatedly observed when $HgCl_2$ was added to cells which were respiring fatty acids. Perhaps this could be explained by a higher permeability of the cells to Hg as the fatty acid salt, resulting in a higher intracellular concentration of Hg^{++}, or to a clogging of the cell membrane by the insoluble complexes formed.

Table II shows the results obtained with various inhibitors when they were tested on purine respiration. It was found that the purines could be used interchangeably in this system except that the use of 6-mercaptopurine was avoided when compounds which are known to react with sulfhydryl groups were used. Not all of the compounds were effective under the same conditions. Thus it was found that rather prolonged pre-incubations with inhibitor were necessary to obtain demonstrable inhibitions with many of the sufhydryl group inhibitors, such as *p*-chloromercuribenzoate, *o*-iodosobenzoate, and iodoacetate. Whenever possible, preincubations with purines were run to obtain internal controls on the purine-induced respiration of the inhibited cells. In addition to the compounds listed in table II the following compounds (maximum concentrations in brackets) showed no significant inhibition on purine-induced respiration of Chlorella:

I. Amino acid metabolism and protein synthesis inhibitors: Methionine sulfoxide (5×10^{-4} M), methionine sulfoximine (10^{-3} M), isonicotinic acid hydrazide (10^{-3} M), azaserine (10^{-4} M), and chloramphenicol (10^{-3} M).

II. Phosphorylation inhibitors: Potassium pyrophosphate (10^{-3} M), and sodium arsenate (10^{-3} M with and without the presence of phosphate buffer).

III. Glycolic acid oxidase inhibitors (19): Disodiumsulfoglycolate (10^{-2} M), disodiumhydroxymethanesulfonate (10^{-2} M), and 2-pyridylhydroxymethane sulfuric acid (10^{-2} M).

IV. Coenzyme A antagonists: Methylpantoyl lactone (5×10^{-3} M) and calcium-D-pantoyl taurine (5×10^{-3} M).

V. Miscellaneous: Sodium malonate (10^{-2} M), imidazole (10^{-3} M), 2-aminoethylthiuronium bromide hydrobromide (3×10^{-3} M), (20), $PbCl_2$ (10^{-4} M), and 3-amino-1,2,4-triazole (10^{-2} M).

Even preincubation periods of several hours failed to reveal any inhibition by the pantothenic acid analogs. An anomalous result was observed with sodium succinate which at 3×10^{-3} M inhibited the stimulation by 6-mercaptopurine by 50 % while not affecting the response to adenine. Apparently the affinity of mercaptopurine for the enzyme site is considerably smaller than that of adenine even though the maximum stimulation which can be obtained with mercaptopurine is almost double that of adenine.

The respiratory quotient for the Chlorella cells in the presence or absence of added purines was consistently 1.0. However, it was found that if nitrogen-starved Chlorella cells were allowed to fix $C^{14}O_2$ overnight and the specific activity of the expired CO_2 determined, a large decrease in specific activity was observed in the presence of the purines. Table III shows the effect of time on the specific activity of the expired CO_2 in the presence and absence of added purines. Manifold repetition of these experiments has shown that the specific activity of the expired CO_2 during the first hour in the purine containing flasks is one-half that of endogenous. This difference tended to decrease in cells which had been incubated on the shaker for increasing lengths of time before respiration was measured, as the specific activity of endogenous respiration tended to decrease with time.

The observed decrease in specific activity of respired CO_2 appears to be characteristic of the purines and not a special effect of 6-mercaptopurine. Table IV shows a comparison of the effects obtained with several different purines. Note that those purines which were more active in stimulating respiration also caused the larger drop in the specific activity of the respired CO_2.

In addition to the inhibitors of the purine effect on respiration which were enumerated above several other compounds were tested, but results could not be interpreted properly because the large stimulation of endogenous respiration which was caused by the in-

TABLE II

ACTION OF VARIOUS INHIBITORS ON PURINE INDUCED RESPIRATION OF CHLORELLA

COMPOUND	CONC. (M)	% CHANGE OF ENDOGENOUS RESPIRATION*	PURINE ADDED**	% INHIBITION***
1. Sulfhydryl Inhibitors				
$CuSO_4$†	10^{-4}	−40	6-Cyanopurine	40
$HgCl_2$†	3×10^{-5}	0	6-Cyanopurine	45
Na arsenite†	10^{-3}	−10	6-Cyanopurine	98
$CdSO_4$††	10^{-4}	0	Purine	73
Na Iodoacetate‡	10^{-3}	0	6-Cyanopurine	60
p-Chloromercuribenzoate‡	5×10^{-5}	+12	6-Cyanopurine	41
o-iodosobenzoate‡	10^{-4}	+15	6-Cyanopurine	62
2. Carbonyl reagents				
$NaHSO_3$††	10^{-4}	+60	Purine	100
Hydroxylamine HCl††	10^{-2}	+18	Purine	98
Semicarbazine · HCl††	10^{-2}	+18	Purine	64
Hydrazine · H_2O††	10^{-2}	−15	Purine	50
3. Amine Oxidase Substrates				
Tyramine · HCl†	10^{-3}	−18	6-Mercaptopurine	11
Tryptamine · HCl†	5×10^{-3}	0	6-Mercaptopurine	52
Histamine · HCl††	5×10^{-3}	+84	6-Mercaptopurine	39
4. Miscellaneous				
Ascorbic acid††	10^{-2}	+ 7	6-Mercaptopurine	100
Na fluoroacetate‡	10^{-4}	0	Purine	15

* Experiments run for 3 hours. Inhibition (−), stimulation (+). Calculation, see table I.
** Purine concentration was 10^{-4} M in all cases.
*** Calculation, see table I.
† Inhibitor and purine were added simultaneously.
†† Inhibitor added after 45 minutes preincubation with purine.
‡ Purine added after 60 minutes preincubation with inhibitor.

TABLE III

VARIATION WITH TIME OF SPECIFIC ACTIVITY OF EXPIRED $C^{14}O_2$ IN 6-MERCAPTOPURINE TREATED & UNTREATED CHLORELLA*

	First hr		1.5–2.5 hr		2.5–3.5 hr		3.75–4.75 hr	
	μl CO_2 flask/hr	Specific activity cpm/μl	μl CO_2 flask/hr	Specific activity cpm/μl	μl CO_2 flask/hr	Specific activity cpm/μl	μl CO_2 flask/hr	Specific activity cpm/μl
No addition	56	1.11	53	1.07	58	0.96	50	0.78
10^{-4} M 6-mercaptopurine**	95	0.48	91	0.58	86	0.60	84	0.56

* Cells were allowed to assimilate $C^{14}O_2$ for 16 hours, washed, and suspended in 0.067 M phosphate buffer, pH 6.0, and shaken in the dark at 25° C. Manometric studies were for 1 hour at the intervals shown, using fresh cells each time. Results are averages of duplicate determinations.

** Mercaptopurine was present from time 0.

TABLE IV

EFFECT OF VARIOUS PURINES ON SPECIFIC ACTIVITY OF RESPIRED $C^{14}O_2$ FROM SHORT-TIME C^{14}-LABELED CHLORELLA*

Compound	μl O_2 Purine / μl O_2 endog.	Specific activ. (endog.) / Specific activ. (purine)
Adenine	2.1	1.6
6-Cyanopurine	3.0	1.9
6-Mercaptopurine	2.9	2.4
6-Chloropurine	1.9	1.3
8-Azaxanthine	2.0	1.6
1-Methylhypoxanthine	1.3	1.1

* CO_2 was collected for 1 hour. See table III for details.

hibitors themselves was not additive with the stimulation caused by the purines. It was hoped that this problem might be resolved by an investigation of the effect of these compounds on the purine-induced depression of the specific activity of respired CO_2 in short-time C^{14}-labeled cells. Previous experiments had shown that a good correlation existed between the inhibition of the purine effect on respiration and the inhibition of the purine effect on specific activity of respired CO_2. Table V shows the results obtained with the compounds which stimulated endogenous respiration. The last compound in the table, purine-6-carboxyhydrazide is included to demonstrate its reversal of the effect of 6-mercaptopurine on the specific activity of the respired CO_2. This compound was previously shown (1) to inhibit the effect of active

TABLE V

EFFECT OF RESPIRATION STIMULATING INHIBITORS ON SPECIFIC ACTIVITY OF RESPIRATORY $C^{14}O_2$ FROM C^{14}-LABELED CHLORELLA CELLS IN PRESENCE OF PURINES*

Compound	Conc	Relative respiration: Endogenous**	Relative respiration: Mercaptopurine***	Specific activity of $C^{14}O_2$ cpm/μl CO_2: Endog.	Specific activity of $C^{14}O_2$ cpm/μl CO_2: Mercaptopurine	Specific activity of $C^{14}O_2$ cpm/μl CO_2: Inhibitor	Inhibitor + mercaptopurine
Na Fluoride	10^{-4} M	2.0	0.83	15.4	8.2	7.3	14.1
2,4-Dinitrophenol	3×10^{-5} M	1.4	0.58	13.9	7.1	7.5	9.0
K Cyanide	10^{-4} M	1.8	0.73	16.4	8.1	13.3	22.1
Na Azide	10^{-3} M	3.2	>1.00†	7.1	4.2	4.0	4.0
Purine-6-carboxhydrazide	10^{-4} M	1.0	0.35	5.1	2.7	4.5	6.9

* Duration of all experiments was 3 hours. Experiments run at 0.067 M phosphate buffer, pH 6, at 25° C in the dark.

** Respiration is recorded as $\frac{\mu l\ O_2 \text{ uptake (with inhibitor)}}{\mu l\ O_2 \text{ uptake (without inhibitor)}}$

*** See table I for calculation. These results are not multiplied by 100.

† Respiration with inhibitor alone was larger than with 6-mercaptopurine, or 6-mercaptopurine plus inhibitor. The latter two respiration rates were identical.

purines in stimulating the respiration of Chlorella. Unfortunately the other compounds by themselves appear to exert a very similar effect on the evolution of CO_2 by the cells as do the purines and thus no conclusions concerning their action on the purine respiration could be drawn.

Discussion

From the inactivity of azaserine, isonicotinic acid hydrazide, and the methionine analogs as inhibitors of the purine effect on respiration, any amination via glutamine or reaction involving pyridoxal phosphate can probably be excluded as a possible explanation of this effect (7, 14, 15). Similarly, the inactivity of the three glycolic acid oxidase inhibitors, disodiumsulfoglycolate, disodiumhydroxymethanesulfonate, and 2-pyridylhydroxymethane sulfuric acid (19), makes the involvement of the two carbon acid cycle unlikely. The inactivity of malonate might well be due to permeability difficulties since it has been shown (8) that the dicarboxylic acids are taken up poorly by Chlorella. Imidazole had been suggested as an inhibitor in view of the activity in our system of several of the inhibitors found by Martin and Stumpf (10) for fatty acid oxidation. The inactivity of pyrophosphate and arsenate, coupled with the questionable results with KCN, NaN_3, and dinitrophenol would tend to eliminate the possibility that the purine effect is the result of an uncoupling of oxidative phosphorylation or a blocking of the terminal stages of the cytochrome chain although the results do not permit the complete elimination of this possibility. Preliminary experiments failed to reveal any decrease in acid-labile phosphate in the cells following exposure to the purines, again supporting this conclusion.

All those compounds which showed inhibitory activity fall into one of two categories: Either they are sulfhydryl inhibitors or compounds which have previously been shown to inhibit or act as substrates for flavoprotein enzymes. The inhibition by arsenite and cadmium supports the possibility that a dimercapto site is involved in the purine effect. Unfortunately this is not very helpful since it is well known that at least two key enzymes in oxidative catabolism —pyruvic and alpha-ketoglutaric dehydrogenase— have such a grouping and are inhibited by these compounds (12). The inhibition by fluoroacetate also points to the involvement of the tricarboxylic acid cycle (5). The carbonyl reagents, bisulfite, hydrazine, hydroxylamine, and semicarbazine have been shown to inhibit such flavine activated enzymes as xanthine oxidase and aldehyde oxidase (16, 17). The observation that the substrates for the flavine-catalyzed amine oxidase, tryptamine, tyramine, and histamine were inhibitors of the purine effect, was unexpected. The rather broad substrate specificity of the flavoprotein oxidases is well known. Thus, xanthine oxidase is also active as an aldehyde oxidase. It may be that the amines served as alternate substrates for the enzyme which acts on the purines, thus preventing an expression of the purine effect.

The specificity of the reaction for purines over pyrimidines, and the complete absence of any lag in the observed stimulation by purines, along with the broad spectrum of activity for purines and purine analogs make it unlikely that these compounds have to be converted to a glycoside or glycoside phosphate for activity. Xanthine oxidase is, perhaps, the only enzyme which uses a wide range of free purines and analogs as substrates, and is a flavoprotein enzyme. Thus xanthine oxidase may form part of the mechanism of the purine effect, but the following considerations must be kept in mind:

I. Many of the purines which were found to be active could not possibly act as substrates for further oxidation by xanthine oxidase for lack of an oxidizable position (azaguanine, azaxanthine, uric acid for example).

II. The oxygen uptake and CO_2 evolution are a multiple of the amounts required for the complete oxidation of the purines. At the same time very little radioactivity was released from C^{14}-labeled 6-mercaptopurine and 8-azaguanine (1, 2) indicating that the purine and triazolopyrimidine rings need not be broken for stimulation to be observed.

In order to reconcile these observations one may assume that a cyclic process is operating in which the purines are alternatively oxidized and reduced. The oxidation of the purines would take place via a xanthine oxidase and would involve no CO_2 liberation. The reduction of the oxidized purines could be coupled to the oxidation of some endogenous substrate which could then be further oxidized by other enzymes in the cell. This might involve the tricarboxylic acid cycle to release CO_2 and take up more oxygen. The reversal of xanthine oxidase has been reported before (18). This interpretation assigns to xanthine oxidase a novel role in a terminal oxidation sequence. Evidence for the reversal of xanthine oxidase in Chlorella, using 8-azaguanine as substrate has been presented elsewhere (3).

Several indirect arguments suggest that the metabolic substrates which are oxidized in this system might be fatty acids. It is known that Chlorella, as well as many other organisms (9), accumulate fat when they are nitrogen-starved. Thus, the most likely respiratory substrate in these tissues is fat. Previous workers have shown (11) that the fat of Chlorella becomes markedly less unsaturated during nitrogen-starvation. At the same time, work with many tissues has shown that fatty acid oxidase is much more active on unsaturated fatty acids than on saturated acids. Our results have confirmed this for Chlorella. Finally, the fat pool is the only major substrate pool which does not reach equilibrium with the rest of the cell in short time photosynthesis experiments (6). Thus, the drop in the specific activity of respired CO_2 in the presence of purines could be interpreted as an increase in the oxidation of the weakly labeled fatty

acids. If it is assumed that the endogenous respiration remains unchanged in the presence of the purines, it can be calculated from our radioactivity results that up to 80 % of the CO_2 which was released in excess of endogenous in the presence of purines must have been derived from a completely unlabeled substrate. Another publication (Bach, M. K. Stimulation of oxidation of neutral lipids in Chlorella by purines. Unpublished.) will present direct evidence for the role of neutral lipids as substrates for the increased respiration caused by purines.

Summary

The action of inhibitors on the previously reported stimulation of endogenous respiration of nitrogen-starved Chlorella cells by purines was studied. Two classes of compounds were found which inhibited purine-induced respiration without affecting endogenous respiration. One group of compounds consisted primarily of sulfhydryl inhibitors, while the second was made up of compounds which are either substrates or inhibitors of known flavoprotein enzymes. Inhibitors of cellular electron transport and oxidative phosphorylation were inactive in this system, or stimulated respiration by themselves. The stimulatory purines caused a marked decrease in the specific activity of respired $C^{14}O_2$ from cells which had been allowed to fix $C^{14}O_2$ for short periods. This drop is interpreted as a stimulation of fatty acid oxidation by the purines. The results are discussed as supporting evidence for the involvement of the purines in a flavoprotein catalyzed oxidation of fatty acids.

Acknowledgments

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STEADY STATE GROWTH OF AVENA COLEOPTILE SECTIONS IN HIGH AUXIN CONCENTRATIONS [1]

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The excised Avena coleoptile section constitutes a convenient system for studying the enlargement of plant cells in response to applied auxin. A portion of the convenience lies in the fact that it has proven possible to arrange conditions such that auxin-induced extension of the section takes place at a rate which is constant with time over periods of several hours. Under these steady state conditions, the powerful methods of chemical kinetics may be applied to the analysis of the interaction of auxin with tissue (4).

The conditions required for steady state extension of Avena coleoptile sections in solutions of suboptimal auxin concentration (less than ca. 10^{-5} M indole-3-acetic acid) have been achieved by Foster, McRae, and Bonner (6), Bennet-Clark and Kefford (2), Bonner and Foster (3), and others. Less general agreement has however been achieved concerning the nature of the response of sections to supraoptimal (ca. 10^{-5} M & higher) concentrations of auxin. Concentrations of auxin up to 10^{-3} M have been reported by Foster et al (6) and by Bonner and Foster (3) to elicit section growth rates which are constant with time but less than that elicited by the optimal concentration. Other investigators including Bennet-Clark (1), Bennet-Clark and Kefford (2), Hancock (7), Housley et al (8) and Marinos (9) find that high auxin concentrations elicit an initial rapid extension, followed by a progressive and continuing decline in extension rate. The present work has been done to try to find any differences in technique which might cause this difference in extension behavior and to discover how to establish reproducible steady state extension rates for coleoptile sections grown in supraoptimal auxin concentrations.

MATERIAL & METHODS

Avena sativa L. variety Siegeshafer from Svalöf was used throughout. The seedlings were grown in one of three different ways.

I. In one type of experiment an attempt was made to reproduce as exactly as possible the conditions used by Foster et al (6). Seeds were soaked for 1 hour in distilled water and then planted in moistened (with distilled water) vermiculite obtained from Pasadena and of North American (Montana) origin.

II. In the second type of experiment, dehusked seeds were soaked for 1 hour in distilled water and then planted on filter paper over distilled water.

III. In the third type of experiment, seeds were soaked for 1 hour in distilled water and then planted in moistened (with distilled water) vermiculite obtained in Canberra and of South African origin. This vermiculite had been either thoroughly washed with water or with a complete Hoagland's nutrient solution.

In all cases germination and growth of the seedlings were allowed to take place in a standard Avena room in red light at 25° C. After 70 hours, coleoptiles 2.5 to 3.0 cm in length were selected and one section 6 mm long was cut 3 mm from the tip of each.

The sections as cut were collected either into distilled water or into a basal nutrient solution consisting of 3 % sucrose and potassium maleate buffer, 2.5×10^{-3} M, pH 4.5. After a minimum period of 30 minutes in the collecting medium, the initial length of each section was measured using a microscope equipped with an ocular micrometer. The sections were then transferred in lots of 20 to the several growth media of the experiment. These media all contained 3 % sucrose, potassium maleate buffer 2.5×10^{-3} M, pH 4.5 and indole-3-acetic acid (IAA) in the concentrations indicated below. Stock solutions of IAA were made up by dissolving in hot water or dilute potassium hydroxide.

Incubation of the sections took place at 25° C in red light. Lengths of the sections were measured at intervals of two to three hours as described above.

Since the amount of North American vermiculite available was small, only one experiment could be done with plants grown according to method I. This was considered sufficient because, as will be shown below, the results were the same as those obtained many times under identical conditions in Pasadena by Bonner and Foster (3, 4). Experiments were done with plants grown according to methods II and III on five occasions for each method.

[1] Received November 14, 1960.

[2] Present address: Division of Biology, California Institute of Technology, Pasadena. The visit during which the present work was done, was supported by the Commonwealth Bank of Australia.

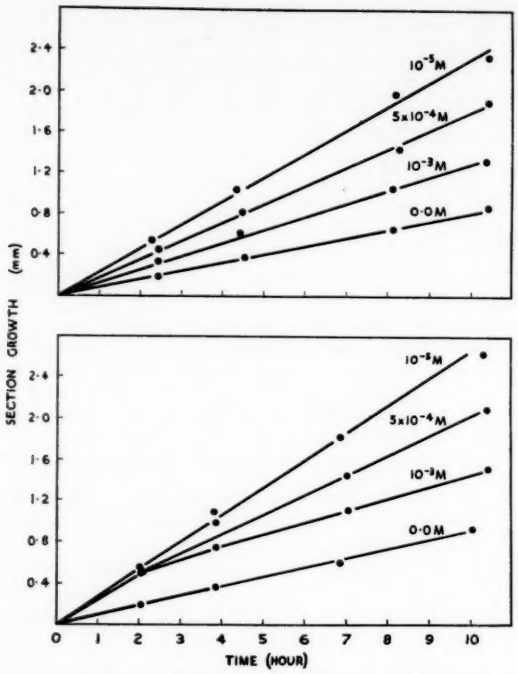

FIG. 1 (*upper*). Progress curves for elongation of coleoptile sections in varied concentrations of indole-3-acetic acid (IAA). Sections from seedlings grown in North American vermiculite moistened with distilled water.

FIG. 2 (*lower*). Progress curves for elongation of coleoptile sections in varied concentrations of IAA. Sections from seedlings grown on filter paper over distilled water.

RESULTS

The data of figure 1 concern an experiment with coleoptile sections produced by method I above and representing, as exactly as possible, a duplication of the material used by Foster et al (6). It is clear that the growth rates of such sections are constant for periods of at least 10 hours. This is true both for sections in media containing IAA in approximately optimal concentration (10^{-5} M) and for sections in media containing supraoptimal IAA concentrations, such as 10^{-3} M. Thus the results of Foster et al (6) and of Bonner and Foster (3) are confirmed.

The data of figure 2 concern an experiment conducted with coleoptile sections cut from seedlings germinated and grown on filter paper over distilled water (method II above). The progress curves of figure 2 show that while the rate of elongation of sections in near-optimal auxin concentration (10^{-5} M) approximates constancy with time from zero time on, the rates of elongation of sections in higher auxin concentrations assume steady and characteristic values only after an initial period of ca. two to four hours. During this initial period, the growth rate of sections in solution containing 10^{-3} M IAA approaches that of sections in near-optimal auxin concentrations (see also 5).

We have at present no explanation to offer for the differences in initial growth rate between these sections and those from seedlings grown according to method I. If one is concerned only with steady state growth rates, it is convenient to regard the initial 2 hour growth period as a pre-incubation during which the sections are immersed in the medium and allowed to equilibrate with it. The progress curves of figure 2 show that the growth rates of coleoptile sections from seedlings germinated in distilled water, are constant with time after the initial 2 hour period and show further that the absolute growth rates elicited are essentially identical to those obtained with sections grown in the same auxin concentrations but cut from seedlings grown by method I.

The growth of sections obtained from plants grown by method III, namely in South African vermiculite equilibrated with distilled water or Hoagland's nutrient solution, in general resembles qualitatively that of sections from plants grown over distilled water. Their extension rates, however, were lower and times required to achieve steady state extension rates longer than for sections from seedlings grown by either of the other two methods.

It has been shown by Foster et al (4), for their experiments, that the steady state growth rate of coleoptile sections becomes inversely proportional to auxin concentration at high auxin concentrations (10^{-5} to 10^{-3} M). This relationship is that predicted on the basis of the concept of two point interaction between auxin and auxin-binding entities of the coleoptile. That the same relationship is found for experiments of the type illustrated in figure 2 is indicated in figure 3.

DISCUSSION

It is now generally agreed (1, 4) that among the more important provisions needed to ensure steady

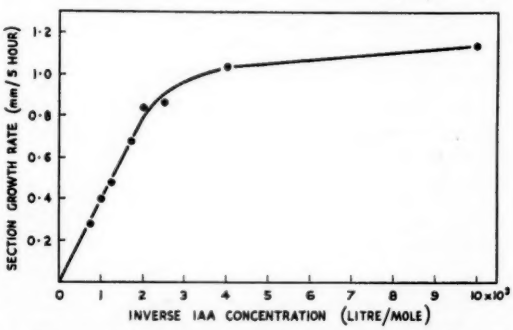

FIG. 3. Steady-state elongation rates of coleoptile sections in concentrations of IAA greater than 10^{-5} M as a function of reciprocal IAA concentration. Sections from seedlings grown on filter paper over distilled water.

state growth of Avena coleoptile sections in optimal or suboptimal auxin concentrations are (4):

I. An external supply of absorbable solutes, such as sucrose, so that the tissue may maintain its internal osmotic concentration.

II. Constancy of external pH, auxin concentration, and other factors.

III. Equilibration of sections in water or growth medium lacking auxin prior to addition of auxin to minimize osmotically induced transient volume changes.

Under these conditions steady state extension is established soon after the sections are immersed in auxin-containing solution. Equilibration of sections with solutions containing low concentrations of auxin is therefore rapid.

The considerations noted above may be expected to apply equally to the maintenance of constant growth rates of sections immersed in solutions of supraoptimal auxin concentration. In fact it has been shown that for sections from seedlings grown in North American vermiculite, steady state extension rates are established quickly in supraoptimal auxin concentrations. Sections from seedlings grown by the other two methods however, behave as if equilibration of the sections with auxin may take longer with high auxin concentrations than with low. The same conclusion may be drawn from the work of Cleland and Bennet-Clark (5).

The present experiments have been concerned with auxin concentrations up to 1×10^{-3} M. Still higher auxin concentrations, as for example 2.5×10^{-3} M or higher, have been found to cause section shrinkage and ultimate loss of turgor, which is observable after 4 to 8 hours of incubation and suggests irreversible damage to the section. Cleland and Bennet-Clark (5) have found that small amounts of alcohol in the test solution lead to the ultimate shrinkage of sections. Some instances of shrinkage reported in the literature may thus be explained by the use of alcohol to bring IAA into solution.

Summary

Progress curves for Avena coleoptile sections incubated in supraoptimal (10^{-5} M & higher) concentrations of the auxin indole-3-acetic acid have been found to be characterized in general by a long period of steady state growth rates preceded in some instances by an initial transient period of more rapid growth. In the present experiments the conditions of growth of the Avena seedlings determined whether coleoptile section steady state growth rates were achieved immediately or after a transition period. In order to establish steady state growth of sections in solutions of supraoptimal auxin concentration it may therefore be necessary to preincubate sections for approximately two hours in basal medium containing auxin in the desired final concentration. When this provision is observed, it is possible to obtain reproducible, steady state growth of Avena coleoptile sections in solutions of high auxin concentration. The steady state growth rates are inversely proportional to auxin concentration as expected on the basis of the two point interaction concept.

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EFFECT OF PHOTOCHEMICALLY PRODUCED OXIDANTS ON GROWTH OF AVENA COLEOPTILE SECTIONS [1, 2]

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An excellent summary of the general effects of both oxidant and non-oxidant type air pollutants on plants is provided in a paper by Middleton et al (4). The effects described are largely concerned with visible pathological symptoms involving the death of cells.

The question of whether or not air pollutants can decrease plant growth without causing cell death has been partially answered in the affirmative by the work of Todd and Garber (9), Hull et al (2), and Koritz and Went (3). Todd and Garber (9) suggested that a direct effect on cell expansion in pea plants might be found. Hull et al (2) found that Avena seedlings which had been exposed to ozonated hydrocarbons showed a decreased response to auxin applied subsequent to exposure. Since the seedlings were composed of cells which may have been undergoing division it is not possible to draw a direct conclusion as to the effect of fumigants on cell expansion.

To determine the effect of oxidants on cell expansion in Avena coleoptiles it is necessary to expose coleoptile sections, which are no longer undergoing cell division, to the air-borne oxidants and to observe the auxin-induced elongation. A study to determine the non-lethal concentrations of oxidants required to inhibit growth is a prerequisite to studying the effect of oxidants in vivo on metabolic reactions associated with expansion.

Furthermore a study of the growth response of Avena coleoptiles to the photochemically produced air pollutants other than ozone is especially required since these substances were not tested in the earlier growth studies. Stephens et al (7) have shown that two of the air pollutant products of the photochemical reaction are ozone and peroxyacetyl nitrate (PAN).

It will be shown below that cell elongation in Avena coleoptiles is inhibited by fumigating the sections with photochemically produced oxidants albeit at concentrations exceeding those found in metropolitan atmospheres to date.

MATERIALS & METHODS

Avena seedlings (variety Siegeshafer) were grown for 84 hours on vermiculite, at 26° C under red-orange light and were watered daily with distilled water. Harvesting of sections and all subsequent operations were carried out in diffuse day light except pretreatments and growth tests, which were performed in darkness.

Coleoptiles of 25 to 35 mm length were selected and 5 mm sections cut 3 mm from the apex were used. In all respiration determinations, leafless coleoptile sections were utilized. Since the presence or absence of the leaf had no effect on the growth results obtained, the growth data presented here are from sections with the leaves left in place.

The general pretreatment consisted of placing about 100 sections for growth test or 40 sections for respiration measurement in 50 ml of solution in a gas washing cylinder. The gas washing cylinder fitted with a standard taper 40/50 cap, was 210 mm high by 40 mm outside diameter and had an extra coarse fritted cylinder at the tip of the gas dispersion tube. The fumigating gas was metered through the solution at a rate of 400 ml per minute. Pretreatment and growth solutions were prepared with redistilled or deionized water and are described in the figure legends.

All pretreatment operations were carried out in a constant temperature carbon filtered greenhouse. Temperatures are given with figure legends.

The fumigating gases used were A, an ultraviolet irradiated mixture of trans-2-butene and NO_2, B, ozone, and C, PAN. The irradiated mixture was produced as described by Taylor et al (8). Ozone was produced by passing dry O_2 over neon tubes whose electrodes were connected to a stabilized high voltage transformer. PAN, produced as described by Stephens et al (7), was dispensed from a nitrogen pressurized stainless steel cylinder.

The irradiated mixture was drawn into the pretreatment solutions by means of a vacuum dynapump. Both the ozone and the PAN were continuously diluted with air in a mixing bottle to provide a known concentration. The diluted ozone or PAN was metered through the gas washing cylinders via a manifold, each culture solution vessel having its own valve and rotameter. The diluting air was provided from the pressure side of a dynapump.

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[2] This investigation was supported in part by the Smoke and Fumes Committee of the American Petroleum Institute.

In all fumigations oxidants were measured on an automatic Beckman oxidant recorder. The oxidant measurement is based on the 20 % buffered KI method (pH 7.2–7.6). The oxidant readings were corrected in the case of PAN by the use of an empirical factor based on an infrared determination of the PAN in the steel cylinder, since it was found that the oxidant recorder registered a constant proportion of the actual PAN present.

After pretreatment, groups of 20 sections were distributed to 20 ml of fresh solutions in petri dishes to observe the subsequent growth rate at 25° C. Each growth curve was based on sections obtained from the same pretreatment vessel. The solutions were prepared with or without indoleacetic acid (IAA) in a concentration of 2 or 5 mg per liter as noted. Length was measured periodically for 48 hours or more by means of a binocular dissecting microscope equipped with a stage micrometer. Each value given in the text or figures is the average of 20 sections. Calculation of the standard error of many groups of sections indicated it to range from ± 0.03 mm for sections growing at minimum rates to ± 0.06 mm for those growing at maximum rates. The least significant differences at the 5 % level were 0.09 to 0.17 mm.

Where little or no growth was found after long periods of incubation with IAA solutions, sections were placed in 0.8 M mannitol or 1.5 M KCl solutions for one to two hours in order to see if they could be plasmolyzed. This period is sufficiently short to preclude deplasmolysis by ion absorption. Plasmolysis indicates integrity of the membrane while inability to be plasmolyzed indicates disruption of the cell and impending or actual death.

Basal respiration, which can be defined as respiration not accompanying either solute absorption or growth (5, 6), was measured in connection with the growth experiments. For this purpose, redistilled or deionized water only was used in the pretreatment vessel and in the Warburg flasks. Duplicate Warburg flasks contained 20 sections in 3 ml of water. Oxygen uptake, which was constant during the period of measurement, was determined for at least 1 hour and the results are presented as per cent of control.

Results

In preliminary experiments of this series, IAA was included in the pretreatment solutions. Since it was found that IAA was inactivated by the oxidants (unpublished work in this laboratory), IAA was added periodically to compensate for the amount inactivated. Inhibition of growth was found under such conditions (as shown in fig 1) where the oxidant

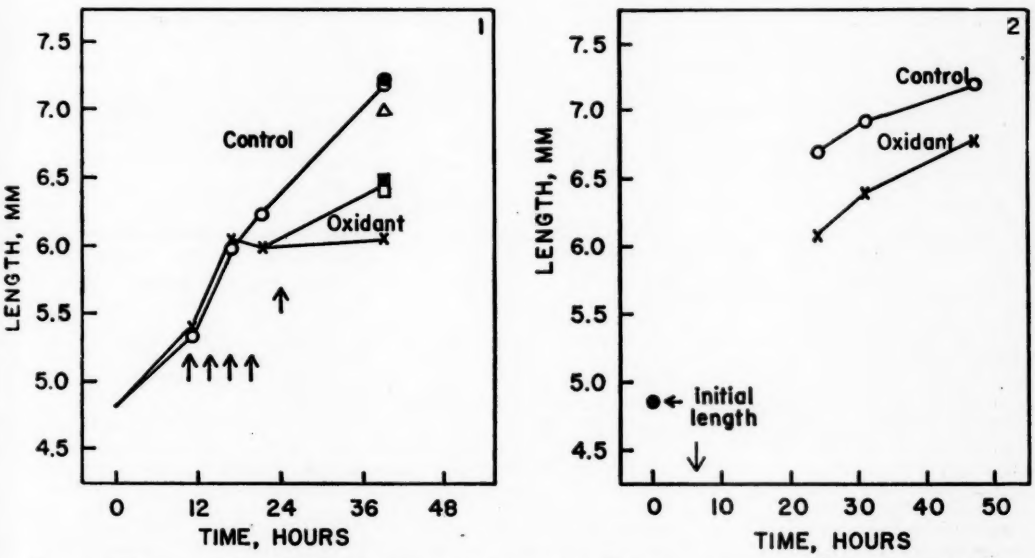

Fig. 1. Effect of oxidant (irradiated mixture of 8 ppm trans-2-butene + 4 ppm NO_2) on length of Avena coleoptile sections as a function of time. Sections fumigated from time zero in 0.025 M KCl + 0.0025 M KH_2PO_4, pH 4.8. One mg per liter IAA added to solutions as indicated by arrows. Last arrow indicates cessation of fumigation and (1) transfer of sections to fresh solution with 5 mg per liter IAA (○, □) (2) the addition of another 1 mg per liter IAA to the pretreatment solutions (△, ■), and (3) no change in solution (●, ×). (oxidant reading = 1.4 ppm). Pretreatment temperature 20 to 21°.

Fig. 2. Effect of oxidant (irradiated mixture of 8 ppm trans-2-butene + 4 ppm NO_2) on length of Avena coleoptile sections as a function of time. Sections fumigated from time zero in 1.5 % sucrose + 0.0025 M KH_2PO_4, pH 4.8. At time indicated by arrow, fumigation was stopped and sections transferred to fresh solutions like the above with the addition of 2 mg per liter IAA. (oxidant reading = 0.8 ppm). Pretreatment temperature = 20° C.

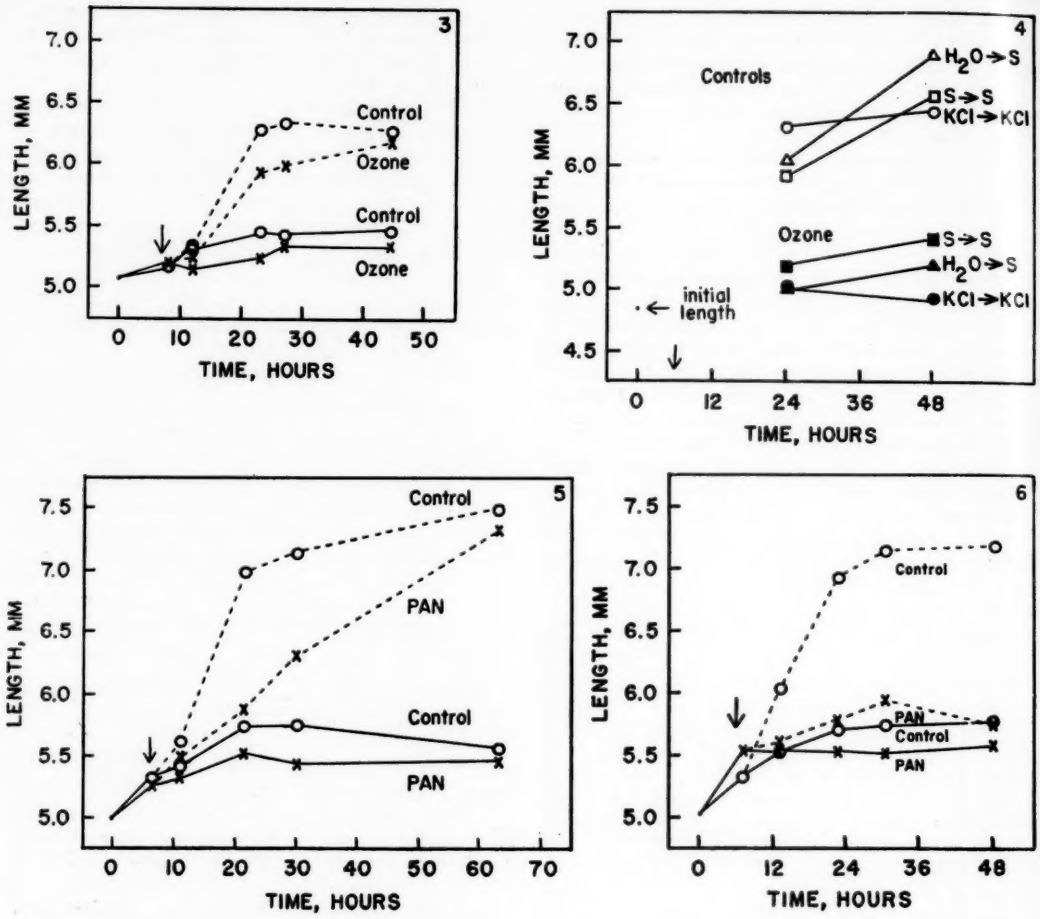

FIG. 3. Effect of 10 to 15 ppm ozone on length of Avena coleoptile sections as a function of time. Sections fumigated from time zero in 0.025 M KCl + 0.0025 M KH_2PO_4, pH 4.8. At time indicated by arrow, fumigation was stopped and sections were transferred to fresh solutions like the above with or without 5 mg per liter IAA. ----- with IAA. ———— without IAA. Pretreatment temperature = 23 to 26° C.

FIG. 4. Effect of 170 ppm ozone on length of Avena coleoptile sections as a function of time. Sections fumigated from time zero either in 0.025 M KCl + 0.0025 M KH_2PO_4, pH 4.8, or in 1.5 % sucrose (S) + 0.0025 M KH_2PO_4, pH 4.8, or in deionized water. At time indicated by arrow, fumigation was stopped and sections were transferred to fresh solutions as indicated with 5 mg per liter IAA. Pretreatment temperature = 24 to 26° C.

FIG. 5. Effect of 1.5 ppm PAN on length of Avena coleoptile sections as a function of time. Sections fumigated from time zero in water. At time indicated by arrow fumigation was stopped and sections were transferred to solutions of 1.5 % sucrose + 0.0025 M KH_2PO_4, pH 4.8, with or without 5 mg per liter IAA. ----- with IAA. ———— without IAA. Pretreatment temperature = 24 to 26° C.

FIG. 6. Effect of 2.3 ppm PAN on length of Avena coleoptile sections as a function of time. Sections fumigated from time zero in 0.025 M KCl + 0.0025 M KH_2PO_4, pH 4.8. At time indicated by arrow, fumigation was stopped and sections were transferred to fresh solutions like the above with or without 5 mg per liter IAA. ---- with IAA. ———— without IAA. Pretreatment temperature = 27° C.

used was that provided by an irradiated mixture which was recorded as 1.4 ppm oxidant (the true oxidant content of irradiated mixtures may have been higher due to only a part of the PAN present being recorded as oxidant). Therefore, because inhibition persisted for at least several hours and it was inconvenient to add IAA periodically, particularly at higher oxidant concentrations, the remainder of the experiments were of the IAA–free pretreatment type.

As shown in figure 2, a 6 hour exposure to an irradiated mixture recording as 0.8 ppm oxidant resulted in about 30 % inhibition of elongation. Although it is apparent that most of the growth occurred during the first 24 hours, growth measurements were extended to 48 hours to see if degree of inhibition would change with time following cessation of fumigation. As figure 2 shows, the inhibition of growth rate disappeared. Basal respiration of sections left in water overnight following pretreatment was not affected.

Irradiated mixtures recording as 0.5 ppm oxidant did not inhibit growth although sections were exposed for 19 hours without IAA. Exposure of sections for 11 hours to irradiated mixtures whose oxidant level was 2.8 ppm caused an irreversible reduction of growth rate and inability of the sections to plasmolyze in KCl or mannitol solutions.

Since the irradiated mixtures contain both ozone and PAN, further experiments were carried out with the pure substances alone. Fumigation with 5 ppm ozone for 19 hours had no effect on subsequent growth. Increasing the ozone concentration to 10 to 15 ppm for 6 hours resulted in a moderate inhibition (fig 3). Basal respiration was not affected. After 20 hours the inhibition of growth rate tended to disappear. To achieve nearly complete growth inhibition it was necessary to expose the sections to 170 ppm ozone for 6 hours (fig 4). The nature of the pretreatment solution, i.e., KCl, sucrose or water, had little effect on this inhibition. Although it was not possible to know in this particular case if growth inhibition was disappearing, the sections could be plasmolyzed after the run was concluded, indicating that the membranes were still intact. From table I it can be seen that basal respiration was slightly stimulated by the ozone pretreatment.

Pretreatment of sections with 0.3 ppm PAN for 6 hours resulted in a slight inhibition of growth. Control sections elongated 1.5 mm and treated sections grew 1.3 mm after 18 hours incubation. A concentration of 1.5 ppm PAN in water exerted a larger inhibition (fig 5). Respiration, however, was unaffected (table I). The tendency for the inhibition to disappear is again apparent in the figure. The use of KCl throughout instead of water in the pretreatment at 1.5 ppm PAN produced similar growth results.

Exposure to 2.8 ppm PAN for 6 hours in the presence of water or sucrose resulted in the subsequent death of many sections. This is probably the lethal concentration for the majority of sections since in another experiment using KCl (fig 6), a slightly lower concentration resulted in severe, non–lethal inhibition. In another experiment, 2.5 ppm PAN treatment inhibited respiration 13 % (table I).

Discussion

The data obtained show that oxidant type air pollutants can interfere with Avena coleoptile growth, thus confirming the findings of Hull et al (*2*). The results clearly show that oxidants directly affect the elongation mechanism.

Furthermore, the photochemically produced oxidants can inhibit cell expansion in a non–lethal manner. The concentrations used have little effect on basal respiration. This is similar to the relative effects of inhibitors on ion absorption and basal respiration in roots (6). The observations tend to support the concept that the oxidants may be specific enzyme inhibitors in the intermediate concentration range as suggested by Freebairn (1).

The very high ozone concentration required to inhibit growth indicates that it was probably PAN which was primarily responsible for the inhibition induced by the irradiated mixtures. The apparent difference in toxicity of ozone and of PAN suggests the existence of two alternative phenomena. Either PAN is much more stable within the tissue than ozone, i.e., ozone may be more readily decomposed on entry into the tissue and therefore a higher external concentration may be required to produce inhibition, or, the mechanisms of inhibition may not be identical. Further work, particularly along biochemical lines, is required to evaluate these alternatives. In passing, it may be noted that unpublished work in this laboratory indicates that IAA in vitro is equally sensitive to both ozone and PAN.

Since the inhibition can occur even when IAA is not limiting, at least in the external solution, it appears that either some step in the growth process is being affected by the oxidants, or, less likely, that the oxidants are being fixed within the cells and are inactivating the incoming IAA.

The range of PAN concentration between non–lethal growth inhibition and death appears to be rather narrow. This may indicate relatively close degrees of sensitivity for metabolic processes involved in growth and those involved in maintaining the cellular membranes. In this connection, Wedding and Erickson (10) showed that permeability of bean tis-

Table I

Effect of Various Oxidant Pretreatments on Subsequent Respiration

Oxidant	% of Aerated control
* Ozone, 170 ppm	118
** PAN, 1.5 ppm	100
*** PAN, 2.5 ppm	87

* Pretreatment temperature 24–26° C
** Pretreatment temperature 24–26° C
*** Pretreatment temperature 26–29° C

sue decreased following exposure to ozonated hexene but increased if exposure time was lengthened.

Although the concentrations dealt with here are about tenfold above those found in the atmosphere (200-fold for ozone) the results may be helpful in the interpretation of mechanisms of growth inhibition in the field. There are probably several reasons why high concentrations of PAN are required to produce growth inhibition in Avena coleoptiles while much lower concentrations affect more sensitive intact plants (unpublished data by Taylor). Although the coleoptile is cutinized and has few stomata, suggesting a transport barrier, there are indications that root tissue which is neither cutinized nor suberized to any great extent may be even less sensitive (work in this laboratory). Thus the answer is more likely to lie either in the resistance of the cellular membranes themselves to the uptake of the oxidants or in the presence of reducing agents in the cell or, simply, less sensitive enzyme systems. Another possibility, namely that the effective concentration of oxidants in the solution is such as to present the plant tissue with a lower level of oxidants than would be expected of tissue exposed directly to the gas phase was ruled out in preliminary work. It was found that intact seedlings in the gas stream were not more sensitive than sections floating in water during gas exposure. The lack of illumination during exposure may also be a factor, since even sensitive petunia plants exposed in the dark to 0.5 ppm oxidant derived from an irradiated mixture were not damaged.

The nature of the recovery mechanism can only be speculated upon. Presumably there might be a slow reduction of oxidized SH groups by endogenously produced reducing agents, provided oxidation has not proceeded too far.

Summary

Avena coleoptile sections incubated in solutions through which photochemically produced oxidant mixtures, ozone or peroxyacetyl nitrate (PAN) are passed, display subsequent repressed growth in fresh solutions to which auxin has been supplied.

Treatment by oxidant recording as 1.4 ppm derived from an irradiated mixture of trans-2-butene and NO_2 results in about 50 % inhibition of elongation. About 1.5 ppm PAN also inhibits growth to the same degree or more. Approximately 170 ppm ozone produced comparable magnitudes of inhibition.

In all cases basal respiration was inhibited little or not at all.

Acknowledgments

We wish to thank Dr. O. C. Taylor, Dr. E. R. Stephens, and Mr. E. A. Cardiff for providing the PAN used in this investigation.

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DIFFUSION OF TRITIATED WATER INTO ROOTS AS INFLUENCED BY WATER STATUS OF TISSUE[1]

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In the course of investigation of physiological responses of plants to water stress it has often been noted that net water uptake declines as moisture stress increases. In unsaturated soils this could be due to depression of moisture flow to the root surfaces. In culture solution, however, the depression can be attributed only to a decline in the permeability of the tissue, either of roots or of leaves, or to a change in the moisture potential difference across the plant.

In recent years Brouwer (2) has cast some doubt on the idea that permeability of the roots declines. He found that in older portions of secondary roots of *Vicia faba* the conductivity to mass flow of water increases when tension in the xylem elements is increased either by suction or by previous treatment at high diffusion pressure deficit (DPD).

Rosene's data (10) also indicate that in onion roots there may be a stress-induced increase in permeability to water, although she thought the increase was due to previous solute uptake which momentarily provided a larger gradient than calculated.

Part of mass flow of water occurs by diffusion mechanism, e.g. as described by Scheidegger (11). Diffusion at pore openings may also govern mass flow as suggested recently by Ray (9). In the case of nonporous membranes all of the mass flow permeability might be measured by diffusive permeability. At boundaries where the resistance to viscous flow is low, a discrepancy may arise and diffusive permeability would not account for the entire mass flow permeability.

Despite this limitation a study of diffusion can help elucidate the mechanism by which water is transferred through living tissue. Accordingly it was decided to determine what effect, if any, water stress had on diffusive permeability of root segments, using tritium oxide as an indicator.

MATERIALS & METHODS

Root segments of *Vicia faba* L., and *Zea mays* L., were used in this investigation. *Vicia faba* (variety Yavne) seedlings were grown as follows. About 120 seeds were soaked overnight in an enameled tray containing a shallow layer of de-ionized water. The seed coats were removed and healthy appearing seeds were planted on water-soaked vermiculite. With more recent batches of vermiculite it was found necessary to soak 2 liters of vermiculite with 2 liters of 0.01 N H_2SO_4 for 1 hour or more. The acid treatment was needed to prevent damage to the primary roots of the Vicia seedlings due to excessive hydroxyl ion concentration. The seeds were placed on the drained and leveled vermiculite and were covered with similar material.

After 3 days in a 25° C dark room, the seedlings were transplanted to aerated one-half strength Hoagland's solution contained in 3 liter glass jars. Four days later the tips of the primary roots were excised and discarded (6). Young secondary roots were harvested 14 days after the seeds were soaked. To obtain older roots the plants were grown an additional week.

The young root material of *Vicia faba* consisted of 10 mm-long segments, cut 3 to 4 mm from the apices of the secondary roots. The diameter of the turgid root material was 0.5 mm. The old material consisted of 12 mm segments (except as noted) cut 75 mm from the apices. The fully turgid diameter was 0.75 mm. About 40 segments of young or 30 of old material were used in each part of each experiment.

About 300 seeds of *Zea mays* (var. single cross Neve Yaar 21) were soaked overnight in the same manner as the Vicia seeds. The seeds were then planted on water-soaked vermiculite (acid treatment was not required). The plants grew 4 days in a 25° C room illuminated by red-orange light. The adventitious roots were harvested and segments 12 mm in length cut 20 mm from the apices were used. The diameter of these roots was 0.9 mm. About 25 segments were used in each vessel. In one experiment, the effect of segment length on diffusion was tested by cutting the 12 mm segments in half. Both Zea and Vicia roots lacked root hairs under these conditions of culture.

The experiments were carried out with approximately 0.1 μc tritiated water (THO) per ml. Groups of root segments were placed in THO solutions for various periods of time. All solutions were prepared with 0.005 M KH_2PO_4 at pH 5. All segments were pretreated for 1 hour in non-radioactive solutions with or without mannitol to attain osmotic equilibrium before transfer to identical solutions containing THO.

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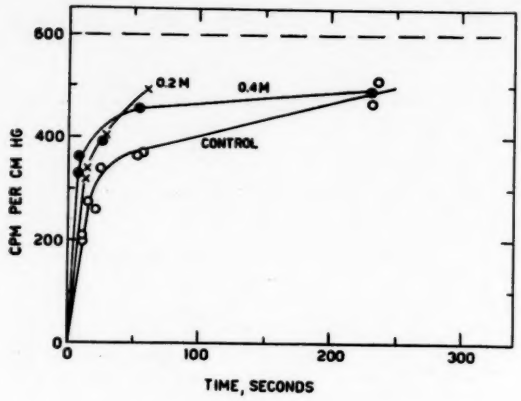

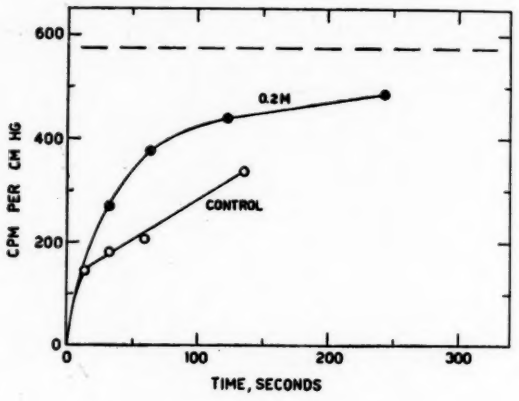

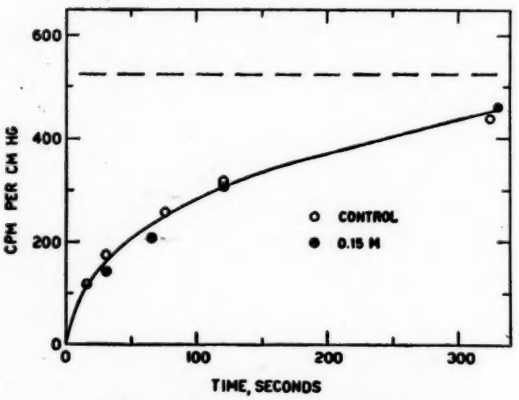

FIG. 1 (*top*). Effect of external osmotic pressure on diffusion of THO into young *Vicia faba* root segments. Dashed line indicates external equilibrium concentration of THO. Solution with various concentrations of mannitol (control = no mannitol), 0.005 M KH_2PO_4, pH 5. Segments pretreated in identical non-radioactive solution.

FIG. 2 (*center*). Effect of external osmotic pressure on diffusion of THO into older *Vicia faba* root segments. Dashed line indicates external equilibrium concentrations of THO. Solutions with or without mannitol 0.005 M KH_2PO_4, pH 5. Segments pretreated in identical non-radioactive solutions.

FIG. 3 (*bottom*). Effect of external osmotic pressure on diffusion of THO into *Zea mays* root segments. Dashed line indicates external equilibrium concentration of THO. Solution with or without mannitol 0.005 M KH_2PO_4, pH 5. Segments pretreated in identical non-radioactive solutions.

The latter solutions were placed in sealed ground glass joint weighing bottles (outside cap type). Experiments were carried out at room temperature. After the desired time interval the segments were poured on a fritted glass plate filter funnel and were dried briefly by suction and blotting by filter paper. The segments were then transferred to a ground glass stoppered vial which was used as the distilling vessel for removal and trapping of water by lyophillization.

The tritium concentration of the trapped water was determined by counting the gas produced after liberation of H_2 and TH by zinc. The data are presented as counts per minute at a pressure of 1 cm Hg, the volume of the Geiger tubes being 250 ml.

RESULTS

As seen in figure 1, in young *Vicia faba* roots which were fully turgid the half time of diffusion ($t_{\frac{1}{2}}$), i.e. the time when the concentration in the roots attains half the concentration of the external solution, was 20 seconds. For tissues placed in plasmolyzing solution of 0.4 M mannitol the half time decreased to 8 seconds. (Incipient plasmolysis for both Vicia and Zea root segments occurred at a concentration equivalent to 0.25 to 0.30 M mannitol solution.)

Since there is the possibility of providing space between cytoplasm and cell wall by plasmolysis which would provide a zone with permeability approaching free self-diffusion, another experiment was carried out at 0.2 M mannitol. The data are also presented in figure 1. Full turgor points are plotted together for both experiments. The decline in half time was almost as great as that found for plasmolyzed tissue.

Since the curves are rather close together just below the half time level, an experiment was carried out with two treatments of five replicates each at 30 seconds. Table I shows that 0.2 M mannitol treatment caused a significant increase in the rate of diffusion.

In segments cut 75 mm from the apices it was found, as shown in figure 2, that the rate of diffusion was increased again by 0.2 M mannitol treatment. By inspection of the curves it appears that the half time was decreased from 105 to 35 seconds.

To see if this increase in diffusion rate occurs in other tissues, experiments were carried out with *Zea mays*. The roots were exposed to moisture stress by placing them in 0.15 M mannitol. In this case no

difference in diffusion rate was found, (fig 3). The half time was 85 seconds.

To compare the permeability data for different tissues and regions a calculation of the diffusion coefficient, D, is required. D may be defined as the quantity of material which diffuses per second across a 1 cm^2 surface under a unit concentration gradient. As suggested by Bonner (1), Fick's second law of diffusion applies to tissue segments, i.e. the segments may be regarded as solid sheets or solid cylinders. As a first approximation one might assume that the major mode of entry of water is through the cylindrical walls with relatively little penetrating the cut surfaces in the time elapsed. This assumption was tested by an experiment on the effect of Zea root segment length on the labeled water content near the half time of diffusion for Zea. If most of the water enters through the radial walls, cylinder length should have no effect on isotope concentration at a given time.

One treatment consisted of 12 mm segments while the other consisted of the same number of 12 mm segments cut in half. Each treatment consisting of five replicates was then immersed in THO for 60 seconds. The difference with 95 % confidence limits was 6 ± 19. Thus there was no significant difference in the diffusion rates and this assumption holds, at least up to a half time in Zea.

Philip (8) has shown how D can be calculated from half time values for diffusion into tissue. In fact the graphical solution of Fick's second law for an infinitely long solid cylinder, such as that plotted by Carslaw and Jaeger (3) in figure 12 of their book for heat flow, can be used to calculate D at any given value of time t, provided the expression $Dt/r^2 = X$ exceeds 0.02. The graph cited above is essentially a plot of $\bar{c}/C$ on the ordinate and X on the abscissa. The general equation for D is

$$\text{I} \qquad D = \frac{Xr^2}{t}$$

where r is the cylinder radius in cm and X is determined by the value of $\bar{c}/C$ at time t (in seconds), $\bar{c}$ being the average inner concentration at time t and C the equilibrium external concentration (which should become the final average inner concentration) ($\bar{c} = 0$ at $t = 0$). For example at $t_{1/2}$, $c/C = 0.5$ and $X = 0.062$.

D should be constant from $0.02 < X < 1.0$ provided new modes of diffusion do not predominate later in time, e.g. slower exchange with a region of bound water.

The radius of the segments was determined as follows. Groups of initially fully turgid Vicia segments were measured and weighed before and after incubation for 1 hour in 0.2 M mannitol. Since the diameter decrease was barely measurable, the calculation of the decrease was made on the basis of weight loss and the assumptions that density = 1 and that length did not change (actually length did decrease ca. 3 %). The average volume of a turgid Vicia root segment 3 mm from the apex was 2.04 mm^3. Initial radius was 0.25 mm. After incubation in mannitol the volume was reduced to 1.92 mm^3. Thus r^2 decreased from 6.25×10^{-4} cm^2 to 5.87×10^{-4} cm^2. Similarly the average volume of a nominally 10 mm segment taken 75 mm from the apex, decreased from 4.05 to 3.79 mm^3. The corresponding values of r^2 were 14.05×10^{-4} and 13.15×10^{-4} cm^2, respectively. Since r^2 was reduced only 7 to 8 % while $t_{1/2}$ was reduced 40 % for young and 65 % for old segments, it is apparent that an increase in D occurred which cannot be accounted for by dimension changes. The change in Zea radius was not determined and the initial value of r^2, 20.25×10^{-4} cm^2 was used in the calculation of D.

A sample calculation of D based on equation I is given in table I. Results of similar calculations using the data of figures 1, 2, and 3 are presented in table II. These calculations give an idea of how well the equation for a cylinder describes diffusion for the tissues examined here. If the final average inner concentration is assumed to be equal to the external concentration, it is evident that D at the terminal points for Vicia, i.e. at 230 to 240 seconds is too low. This indicates the presence of a bound water compartment. The data agree quite well with the equation in the case of Zea. If we assume that the final inner concentration for all treatments of Vicia was 490 cpm, which appears to be the internal equilibrium value for the first phase of diffusion, then the values of D are more or less constant for the time period examined (table II).

In Zea root segments the diffusion coefficient is decreased to less than 10 % of the coefficient in free water using Wang's value of 2.57×10^{-5} cm^2 sec^{-1} at 25° C (13). The apparent diffusion constants for the more rapid water movement phase in Vicia roots are of the same order of magnitude. The younger Vicia roots are about twice as permeable as the older and, as was apparent from the figures, the increase due to turgor decrease is similar in both regions.

TABLE I

EFFECT OF EXTERNAL MANNITOL CONCENTRATION ON THO CONCENTRATION IN TISSUE WATER OF VICIA FABA ROOT SEGMENTS & CALCULATION OF D USING GRAPHIC SOLUTION

	t sec	$\bar{c}$ cpm	$\bar{c}/C$	*X	**D X 10^5 cm^2/sec
Control	30	352	0.569	0.085	0.18
0.2 M Mannitol	30	463	0.780	0.195	0.38
***Difference	...	111 ± 22	...	...	...

* From graph plotted by Carslaw and Jaeger (3).
** See equation I.
*** 95 % Confidence limits.
C = 619 cpm in external solution of control (no mannitol).
C = 594 cpm in external solution of mannitol.
r^2 of Control = 6.25×10^{-4} cm^2.
r^2 of Mannitol treatment = $5.87 \times 10^{-4} cm^2$.

Discussion

Although Philip (7) recently implied that self-diffusion measurements cannot be compared with mass flow permeability determinations, it should be pointed out that his analyses assume an osmotic permeability measurement which involves changes in volume of cells or tissues and the propagation of these changes. The use of the potometer eliminates such changes and theoretically if potential gradients can be determined, a direct comparison of diffusive and mass flow permeabilities should be valid. The work of Mees and Weatherley (5) and of Brouwer (2) indicate that mass flow is sensitive to relatively high concentrations of metabolic inhibitors. Apparently metabolic inhibitors also have some depressing effect on diffusion of deuterium hydroxide into Vicia root segments as shown earlier by Ordin and Kramer (6).

The diffusive permeability observations made here agree with the mass flow permeability data of Brouwer with respect to direction of changes. However, there is a discrepancy between the relative values of permeability for young and old root zones. Since the older portion of the root is thicker than the young portion and the conductivity measurement of Brouwer does not take into account moisture potential gradients, his calculated conductivity may be lower than it should be. However, if the pores were smaller in the older portion, this might help account for the discrepancy between Brouwer's results and those reported here since pore size would have a greater effect on mass flow than on diffusion. As for the permeability increase induced by moisture stress, Brouwer suggested that it is due to a decrease in the pressure exerted on the cytoplasm, i.e., less cell wall-cytoplasm interaction.

Mees and Weatherley (5) found that increased hydrostatic pressure on the external medium surrounding the roots of tomato plants resulted in apparent increases in mass flow permeability while increased external osmotic pressure depressed it. If their suggestion that both high turgor pressure and

Table II

Diffusion Coefficients of Water in Root Segments as a Function of Distance From Apex, of External Mannitol Concentration & of Time

Root	mm From apex	Mannitol conc M	t sec	D X 10^5 cm^2/sec		
				+	++	‡Avg.
*Vicia	3–4	0	10	0.19	0.25	0.27
			14	0.22	0.36	
			20	0.14	0.22	
			24	0.22	0.36	
			54	0.13	0.20	
			233	0.05	0.21	
*Vicia	3–4	0.2	14	0.34	0.54	0.50
			29	0.26	0.46	
			59	0.23		
**Vicia	75	0	32	0.11	0.15	0.13
			59	0.07	0.11	
			135	0.09	0.14	
**Vicia	75	0.2	31	0.23	0.34	0.36
			63	0.25	0.40	
			122	0.21	0.36	
			241	0.14	0.35	
***Zea	20	0	75	0.16		0.16
			120	0.17		
			325	0.16		
***Zea	20	0.15	65	0.12		0.15
			120	0.15		
			330	0.18		

* Data of figure 1: C = 600 cpm; $r^2 = 6.25\times10^{-4}$ and 5.87×10^{-4} cm^2, respectively.
** Data of figure 2: C = 575 cpm; $r^2 = 14.05\times10^{-4}$ and 13.15×10^{-4} cm^2, respectively.
*** Data of figure 3: C = 525 cpm; $r^2 = 20.25\times10^{-4}$ cm^2 for both.
\+ From experimental points with C as indicated above.
++ From experimental points with C at 490 cpm for Vicia only.
‡ Average based on C = 490 cpm for Vicia and on C = 525 for Zea.

high external hydrostatic pressure increase cytoplasm-cell wall interaction is correct, then Brouwer's explanation would appear to be unsatisfactory. Although their results contradict Brouwer's findings and conclusions, it may merely indicate that there are differences in the roots of the two plants which affect water movement.

Hylmö (4) proposed that increased xylem tension in *Vicia faba* may cause smaller pores, already present, to become available for mass flow. Since these pores would always be available for diffusion, this explanation is not adequate.

A reduction of turgor pressure might also result in increased diffusive and mass flow permeability by a reduction of tortuosity of the water pathways. This concept is described by Scheidegger (11). Wang (13) suggests a similar concept which explains a reduction of apparent diffusion constant in protein solutions. He calls it an obstruction effect of the large protein molecules on the water molecules causing the latter to traverse longer paths than expected. On the face of it, Wang's data would suggest increased tortuosity as turgor decreased due to water loss, if cytoplasm were to be considered as a protein solution. It is conceivable, however, that in Vicia a decrease in turgor could lead to realignment of structures or of macromolecules in the cytoplasm with consequent decrease in tortuosity. Alternatively the macromolecules may undergo reduction in volume through partial dehydration again resulting in decreased tortuosity. If the actual path could be measured it might be found that no change at all occurs in the value of D as turgor decreases. In Zea on the other hand, no such change may occur in the cytoplasm (the cell constituents of the two plants need not have the same physical-chemical properties) and as turgor decreases D changes very little.

Summary

Self-diffusion of tritiated water into young *Vicia faba* L. root segments is increased by increasing water stress. Older *Vicia faba* roots are less permeable to tritium hydroxide and show a similar response to water stress.

In corn roots an increase in stress has little or no effect on rate of diffusion.

Exchange of water occurs primarily through the cylindrical walls of root segments rather than through cut surfaces.

Diffusion constants are reduced to about 10 % of that of free self diffusion in pure water.

The results in general appear to agree with those obtained by other workers using mass flow techniques.

A tortuosity factor may be invoked to help explain changes in self-diffusion constants.

Acknowledgments

We wish to thank Dr. A. Nir of the Isotope Department of the Weizmann Institute of Science for his helpful counsel and for supplying the THO. Part of the tritium analyses were carried out by Mrs. B. Mandelbaum of the above department.

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CELL WALL CARBOHYDRATES IN TOBACCO PITH PARENCHYMA AS AFFECTED BY BORON DEFICIENCY & BY GROWTH IN TISSUE CULTURE [1, 2]

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The primary wall of a growing plant cell consists of a mixture of cellulose, pectic substances, hemicelluloses, and protein, and is often isolated with varying amounts of cytoplasmic constituents (13). Current theories of the mechanism of expansion of primary walls are often based on considerations of the properties and orientation of the cellulose micelles. This results largely from an inability to determine the orientation and relationships of the non-cellulosic components of the wall. Kerr (10) suggested that the pectic substances might be responsible for the properties of the primary wall. Roelofsen and Kreger (16, 17) were able to detect some orientation of the pectin in collenchyma of *Petasites vulgaris*. The incubation of Avena coleoptile tissue with IAA caused an increased incorporation of glucose (1) and of methyl ester (7) into the cold water soluble pectin fraction.

It is well established that boron deficiency affects carbohydrate metabolism, leading to an accumulation of carbohydrates in certain parts of the plant (5). Spurr (18) reported that parenchyma cell walls of celery were thickened under conditions of boron deficiency. Winfield (24) suggested that boron is related to pectin synthesis in *Penicillium glaucum*. Boron deficiency has been reported to cause increases in pectin content in sunflower tissue (2) and bean leaves (3). Odlhoff (14) found a slight increase in the concentration of several fractions of cell wall material from the roots, stems, and leaves of boron-deficient bean seedlings. Whittington (19) presented the hypothesis that boron may be concerned with the formation of pectin from uridinediphosphate–D–glucose, and that abnormalities in the formation of the cell wall may prevent the cell from becoming organized for mitosis.

Determinations of the composition of plant cell walls are often made by a series of empirical extractions and precipitations, making it difficult to compare the results of different workers. The difficulties involved are discussed by Jermyn (8) and by Williams and Bevenue (20). Jermyn and Isherwood (9) hydrolyzed the polysaccharides of pear fruit cell walls after preliminary separations, then calculated the amount of each polysaccharide as if it consisted of only a single sugar. This approximation is perhaps better in the cases of cellulose and xylan than for arabans and galactans, and would depend upon the nature of the hemicelluloses in the species being examined.

The present investigation grew out of earlier work on pectin synthesis in tobacco pith tissue (23). First, the effects of boron deficiency on the pectic substances of the cell wall were studied. When it became apparent that there was little relative change in the pectic substances, but a great change in the total amount of wall material, the investigation was broadened to include analyses of the other wall polysaccharides. It will be shown that the wall components which underwent the greatest relative changes were the galactans and the arabans. The former decreased in pith of boron-deficient plants and the latter increased when growth of pith tissue cultures was stimulated by IAA.

MATERIALS & METHODS

Tobacco (*Nicotiana tabacum*, L., Wisconsin var. no. 38) was grown in sand culture in 2-gallon crocks (15). The nutrient solution, applied daily, consisted of: 0.001 M KH_2PO_4, 0.002 M $MgSO_4$, 0.003 M $Ca(NO_3)_2$, 0.0015 M $CaCl_2$, 0.0015 M K_2SO_4, 0.0005 M $(NH_4)_2SO_4$, 1 ppm Fe as $FeSO_4$, 0.25 ppm Mn as $MnCl_2$, 0.01 ppm Zn as $ZnSO_4$, 0.01 ppm Mo as Na_2MoO_4, and 0.01 ppm Cu as $CuSO_4$. The nitrate level was raised in the summer and lowered in the winter. The control plants and all young seedlings received 0.1 ppm B as H_3BO_3. When a plant was to be given a lower level of boron the sand was first flushed with tap water, then the appropriate solution was applied. The low boron levels were usually started when the stems were either about 5 cm or 15 to 20 cm tall, giving deficiency periods of different

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lengths before harvest. Pith tissue was taken from 5 cm below the stem tip to the point at which the stem became woody. Yields of pith of 25 to 50 g fresh weight per plant were obtained, with the higher yields from the control plants. Dry weights were determined on samples of the fresh pith.

Tobacco pith tissue cultures were made by the method of Jablonski and Skoog (6). Sections of pith 15 × 5 × 1.5 mm were cut from the stem and transferred with sterile technique onto 50 ml of a modified White's medium. Expansion of the tissue without cell division was stimulated by 2 ppm IAA in the medium.

The cell wall fraction was prepared by dropping slices of pith tissue into boiling 95% ethanol, with the final concentration being 70% (w/w). The tissue was ground in a VirTis homogenizer at top speed and then subjected to gentle boiling for 10 minutes. The homogenate was filtered and washed successively with 70%, 95%, and absolute ethanol, and finally with ethyl ether. The white, fluffy residue was air-dried, and, in later experiments, stored in a desiccator. The water content of the dried cell wall was determined as the loss in weight after drying at 105° C. The adsorbed water content of one sample of air-dried cell wall changed from 10% to 13% when exposed to relative humidities of 45% and 85%, respectively. After storage in a desiccator the water content was reduced to a lower, less variable level. The dry residue was designated as the "cell wall", though it undoubtably contained cytoplasmic constituents such as protein. The analyses were performed on this dry cell wall fraction.

The pectic substances were determined by the pectinase-carbazole method of McCready and McComb (11).

The other wall polysaccharides were estimated by hydrolyzing the wall and determining the resulting monosaccharides. Fifty mg of cell wall were dissolved in 1 ml of 72% H_2SO_4, which was then diluted with 22.8 ml of water to give a 1.0 N solution. This solution was refluxed for 6 hours. Barium carbonate or barium hydroxide was added to neutrality and the barium sulfate was removed by filtration or centrifugation. The solution was then passed through a small column of Amberlite IR-120(H^+). The solution was concentrated under vacuum and made up to a final volume of 2 ml.

Glucose, galactose, arabinose, xylose, and rhamnose in the hydrolysate were determined by quantitative paper chromatography (22). The sugars were identified only by comparison with the R_f values and colors of known sugars on the same chromatograms. Some mannose was present, but it could not be separated from arabinose. The anhydrous sugars (hexose or pentose units minus one molecule of water) are reported in terms of the corresponding polysaccharide as per cent of the cell wall.

Nitrogen was determined by a micro-Kjeldahl method (21). Protein was calculated as 6.25 × N.

TABLE I

ANALYSIS OF STEM PITH TISSUE FROM CONTROL & BORON DEFICIENT TOBACCO PLANTS

	EXPERIMENT I*			EXPERIMENT II**					
	CONTROL	LOW B		UPPER THIRD		MIDDLE THIRD		LOWER THIRD	
		A	B	CONTROL	LOW B	CONTROL	LOW B	CONTROL	LOW B
Stem ht, cm	90	60	50	47	39	47	39	47	39
Dry wt of pith, %***				5.1	5.3	4.7	5.1	5.0	4.8
Cell wall, %***	1.16	1.32	2.27	0.96	1.97	1.04	1.89	1.67	1.80
Cell wall composition: †									
Cellulose	29	31	32	24	26	28	28	30	30
Pectic substances	20	22	19	18	23	22	22	22	23
Galactan	7.7	6.1	4.7	10.0	7.4	9.2	5.0	6.1	4.2
Araban	3.9	5.0	4.7	5.1	6.0	4.0	4.2	4.7	5.4
Xylan	1.6	1.8	3.1	1.9	2.4	1.5	1.8	1.6	1.7
Rhamnose	1.7	1.5	1.4	1.9	1.6	1.5	1.2	1.7	1.6
Protein	10.5	9.7	8.4	17.4	14.9	11.5	10.4	7.8	10.1
Ash				6.2	4.9	7.9	6.0	9.8	6.9
Water				9.9	10.2	4.3	4.1	5.3	3.1
Unaccounted for	26	23	26	6	4	10	17	11	15

* Experiment I: seed planted March 13, 1956; treatment A given 0.03 ppm boron from June 9 to July 1; treatments A and B given 0.02 ppm boron from July 2 to harvest on July 17. Three plants in each treatment.

** Experiment II: seed planted September 16, 1957; 0.01 ppm boron supplied to low B plants from November 20 to December 19; 0.001 ppm boron supplied from December 20 to harvest on January 2, 1958. Four plants in each treatment. Stems from sand to just below the tip divided into three sections.

*** Fresh weight basis.

† As per cent of cell wall.

Crude ash was determined as the residue after ashing a 100 mg sample of the cell wall for 2 hours at 580° C.

Results

I. Boron Deficiency Symptoms: Moderate boron deficiencies were induced by boron concentrations of 0 to 0.02 ppm in the nutrient medium so that the deficient plants grew nearly as rapidly as the control plants during the period of most rapid growth (from 15 cm high to about 50 cm). If deficiency symptoms appeared very early the boron concentration was raised. Boron deficiency symptoms appeared rapidly in the summer, when the plants grew as much as 4 cm/day, and slowly in the winter. A level of 0.02 ppm boron caused deficiency symptoms to appear in the summer, but the level had to be reduced to 0.001 ppm to produce appreciable symptoms in the winter (Experiments I & II, table I).

Wrinkled leaves and shorter stems were characteristics of all deficient plants when harvested, and the stem tip was usually found to be dead. Elongation of the upper portion of the vegetative axis and inflorescence on the control plants was responsible for most of the height differences. When deficient plants were harvested the vascular tissue and sometimes the pith tissue turned brown upon exposure to air. The per cent dry weight of the pith tissue was not affected by boron deficiency.

II. Cell Wall Polysaccharides: Tests for starch by I_2–KI staining were negative in almost every case, and the glucose found is assumed to come mostly from cellulose. This was further confirmed by a 12-hour hydrolysis in 0.33 N H_2SO_4 with and without first dissolving the cellulose in 72 % H_2SO_4. Only 7 % of the glucose was released if the cellulose was not first dissolved, compared to 60 to 90 % yields of the other sugars.

Maximum yields of most sugars were obtained with a 5-hour hydrolysis in 1 N H_2SO_4, with destruction of the sugars being apparent after 18 hours. Rhamnose behaved differently, with increased amounts being recovered as the hydrolysis time was increased up to 18 hours. This suggests that the rhamnose units are found in the interior of a polysaccharide chain or in such a position that aldobiuronic acids form during hydrolysis. The rhamnose values reported are thus less than the true values.

A typical composition of tobacco pith cell walls is shown by the control tissue of Experiments I and II, table I. Cellulose makes up 25 to 30 % of the cell wall, with the pectic substances accounting for about 20 %. Both of these polymers are present in lesser amounts in the younger tissues. The galactans make up 10 % of the younger walls, but considerably less than that in the older walls. The arabans make up about 5 % of the wall, with the xylans and rhamnose each accounting for less than 2 %. Very small amounts of mannose and ribose were also detected but not estimated. More protein is found in the younger wall fraction than in the older wall, but the protein may not be a true part of the wall. The ash content is variable and also may not be true wall material. The older tissue had a large amount of wall material on a fresh or dry weight basis, so the decrease in galactan percentage from young to old tissue represents a dilution by increased laying down of cellulose and pectic substances.

III. Effects of Boron Deficiency on Cell Walls: Table I gives the results of two typical experiments with boron deficiency. In Experiment I the plants (B) which were grown with 0.02 ppm boron for only 15 days were in a rapidly growing stage when boron was first withheld and were more severely affected than the plants (A) grown for 38 days with 0.03 and 0.02 ppm boron.

The most prominent effect of boron deficiency is a doubling of the amount of the cell wall fraction on both fresh- and dry-weight bases. During harvest it was obvious that the boron-deficient tissue was different from the control tissue—it was harder to cut and harder to homogenize. When sections of pith from the top third of a control stem were placed on moistened filter paper they expanded 25 % in length within a few hours. Sections from more mature tissue expanded to a lesser extent, and sections from all parts of boron-deficient plants expanded less than 10 % in length.

Boron deficiency had little effect on the proportions of the two major components of the wall, cellulose and the pectic substances. The one consistent change in these and other experiments was the decrease (as much as 40 %) in galactans. There was some increase in the arabans, and a doubling of xylans was found in Experiment I, but these changes were often small or inconsistent in other experiments.

In one experiment, plants subjected to nitrogen deficiency were also analyzed. The galactan content of the nitrogen-deficient plants was 8.6 % compared to 6.5 % in the control plants and 4.1 % in the boron-deficient plants. Both deficiencies caused an increase in the amount of wall material on a fresh-weight basis.

The content of soluble sugars (not reported) did not follow a consistent pattern in two boron-deficiency experiments in which it was measured.

IV. Pith Tissue Cultures: Tobacco pith tissue cultures undergo rapid cell enlargement without cell division when supplied with IAA (6). The fresh weight, dry weight, cell wall weight, and amount of pectic substances increase by several hundred per cent over a period of 2 or 3 weeks, with rapid growth commencing 4 to 6 days after the tissue is transplanted (24). The cultures without IAA cease growing and turn brown after about six days. The results obtained in the present study were similar to those reported earlier. In addition, cultures were made with pith from boron-deficient plants. These sections did not expand in length as much as those from normal plants, yet the overall increases in fresh weight, dry

TABLE II

ANALYSIS OF TOBACCO PITH TISSUE GROWN IN STERILE CULTURE WITH & WITHOUT ADDED IAA

	CONTROL	+ IAA			− IAA	
	0 DAY	3 DAY	6 DAY	14 DAY	3 DAY	6 DAY
No. sections*	127	128	96	76	120	92
Fresh wt**	257	303	433	683	288	297
Cell wall**	2.34	4.47	6.44	10.00	3.81	4.29
Cell wall composition: ***						
Cellulose	29	25	23	22	27	25
Pectic substances	26	23	21	23	23	21
Galactan	9.6	8.6	8.3	8.4	8.4	7.3
Araban	4.3	4.8	5.8	7.4	5.2	5.4
Xylan	1.8	1.9	2.0	2.3	2.0	1.7
Rhamnose	1.3	1.3	1.1	1.1	1.4	1.3
Lignin	...	...	...	2.4	3.4	9.4
Protein	10.9	15.1	14.1	13.8	13.2	15.0
Ash	9.3	8.8	8.3	10.6	10.5	9.1
Water	2.0	1.3	2.1	1.3	1.3	1.2
Unaccounted for	6	11	13	7	5	4

* Sections from two experiments started 2 days apart were combined to give enough material for analysis.
** Mg per section.
*** As per cent of cell wall.

weight, and pectic substances were comparable with those in normal tissue. Although tissue was cultured on media prepared with and without boron, no noticeable growth effects were observed. The pyrex flasks used may have supplied an adequate level of boron.

In table II are the results of a tissue culture experiment on pith from control plants. In the rapidly growing IAA-stimulated tissue the relative amount of cellulose decreased by a quarter, while the pectic substances decreased slightly. (No changes in amount of pectic substances were found in other experiments.) The greatest relative change was found in the arabans, which increased from 4.3 to 7.4 % of the wall. On a weight-per-section basis all components of the wall increased rapidly. After 3 days the composition of the untreated cultures was similar to that of the IAA-treated cultures, but between 3 and 6 days there was very little increase in the per section weight of the various polysaccharides in the untreated cultures. After hydrolysis of the untreated tissue, and also the 14-day treated tissue, an undissolved yellow residue which remained was filtered off and weighed. This new component of the wall accounted for a large part of the increase in weight of the untreated tissue between 3 and 6 days. Because of its insolubility in 72 % H_2SO_4 and resistance to hydrolysis, the yellow residue is presumed to be lignin. Phloroglucinol tests for lignin were not done on these tissues, but positive results were obtained on 30-day IAA-treated tissue cultures and also on tissue from boron-deficient plants, indicating that lignin is present in pith tissue under some conditions.

DISCUSSION

The differences in cell wall composition between the pith of normal and boron-deficient plants were similar to the differences between young pith tissue in the upper third of the stem and the older tissue near the base (table I). The largest difference was in the amount of cell wall material on a fresh-weight basis, which was about one per cent in young normal tissue and about double that in both boron-deficient young tissue and in older normal tissue. Along with the increase in cell wall material, the tissue became tougher and less able to expand upon being cut free of the stem. Generally, there were few changes in the relative amounts of cellulose and the pectic substances, which together make up about half of the total cell wall. The one exception was the young tissue, which had markedly less cellulose and pectic substances. In the young tissue, boron deficiency increased the content of both these components over that of the normal tissue. The similarity between boron deficiency and maturation may be due in part to the fact that the samples from the deficient plants would contain less young tissue because of the stem tip necrosis. The consistent reduction of the galactans in all parts of the deficient stems in all experiments conducted indicates that this would not account for the entire effect.

If the composition of the wall is considered as per cent of the wall fraction itself, it would be concluded that boron deficiency specifically inhibits the synthesis of galactan. However, the great increase in total cell wall material on a fresh weight basis means that boron deficiency really stimulated the

synthesis of all the polysaccharides except galactan. On a fresh weight basis the galactan content sometimes increased, sometimes decreased with boron deficiency. The net result is that a boron-deficient plant has pith cell walls containing a lower proportion of galactan compared to the control plant.

The ratio of cellulose to pectic substances and the percentage of cellulose in the wall reached their lowest points of all tissues examined in the 14-day IAA-treated tissue culture. Thus cellulose is found to be low in both the actively growing tissue cultures and the young tissue still in the plant.

The relative cell wall contents of galactan and araban appeared to be most responsive to treatment. Galactans were invariably lower in boron-deficient cell walls than in the corresponding normal cell walls, and were also lower in old as compared to young cell walls.

Although nitrogen deficiency results in the accumulation of carbohydrates and a thickening of the wall, as does boron deficiency, the galactan content in the nitrogen-deficient plants was higher than normal rather than lower. The arabans did not change with the galactans, but instead increased to a high value only in the expanding tissue cultures. The galactans and arabans together usually accounted for about 12 % of the wall, with a maximum of 17 % being found in a sample of young tissue and a minimum of 8.3 % in a boron-deficient tissue. The ratio of galactan to araban varied from a high of 2.3 : 1 to 0.8 : 1.

The pectic substances can be divided into various fractions according to solubility, and, when isolated, the fractions usually contain galactans and arabans as well as polyuronic acid. Microfibers made up of galacturonic acid, galactose, and arabinose have been isolated from tobacco pith tissue (12). Fuller (4), using a strip electrophoresis method, was able to divide the pectic substances in elongating bean roots into three fractions. One fraction, thought to be a galactan, increased in content per cell up to a distance of 43 mm from the tip, then remained constant, whereas a second fraction increased only during elongation. Several combinations of polysaccharides might account for the present results. The pectic triad might consist of several discrete fractions, each with a certain proportion of polygalacturonic acid, galactan, and araban; and the relative amount of each fraction could vary with age and physiological condition. On the other hand, the proportion of each of the three components of the pectic combination might vary over a broad spectrum under different conditions. Finally, a part of the galactans and arabans may be independent of the pectic substances and each other. The present work and that of Fuller (4) indicate that the three components of the pectic fraction change in relative amount with changing physiological conditions in growing primary cell walls, and that further studies are needed to determine if the properties of the primary wall may be dependent upon these polysaccharides rather than upon cellulose.

Summary

The composition of the cell wall of tobacco pith tissue was determined by hydrolyzing the wall and determining the sugars released. The sugars were reported as if they were derived from polysaccharides made up of single sugars. The cell wall fraction typically contained 25 to 30 % cellulose, about 20 % pectic substances, 8 % galactan, 5 % araban, and less than 2 % xylan and rhamnose. The isolated wall also contained variable amounts of protein, ash, and adsorbed water. The walls from young tissue contained less cellulose and less pectic substances, but more galactan than mature tissue.

Boron deficiency caused a marked increase in the total amount of cell wall material accompanied by a drop in the relative galactan content. Boron deficiency caused no great shift in the relative amounts of either the pectic substances or cellulose, except that the amounts in young deficient tissue were increased toward the levels found in mature normal tissue.

Pith tissue in sterile culture was stimulated to expand without cell division by adding IAA to the medium. Cell walls from tissues which had more than doubled in size contained a lower proportion of cellulose while the percentage of araban was increased.

The cellulose content was lowest in growing tissues. The relatively great changes in galactan and araban contents suggested that changes in these polysaccharides might be related to the physiological condition of the primary cell wall.

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ON THE MASS CULTURE OF ALGAE. III. LIGHT DIFFUSERS; HIGH VS LOW TEMPERATURE CHLORELLAS[1, 2]

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The present work is introduced by a previous report (4). The yield of algae under sunlight irradiance is limited by the characteristic of light-saturation. The Chlorellas and all other algae studied to date become light-saturated at values of irradiance far less than that of full sunlight. The resulting efficiency of light utilization is considerably lower than that achievable under low incident irradiance. For mass culture under sunlight illumination attempts to increase yield become attempts to minimize or circumvent the limitations due to light-saturation. The present work is concerned with two possible approaches: A, the use of a cone diffuser as a physical means of light attenuation and B, the use of an alga with a higher temperature optimum and a presumed higher irradiance for light-saturation.

METHODS

We have chosen to study yield (mg/day) in cultures of Chlorella maintained under controllable conditions in the laboratory. Most of the methods used have been described in detail (4). Continuous light from a tungsten lamp, filtered through water and copper sulfate, was presented to the open horizontal surface of a culture as an approximately collimated beam of irradiance equivalent to full sunlight. The culture vessel was a flat-bottomed glass cylinder of 67 mm ID containing 1,010 ml of algal suspension at a normal working depth of 267 mm. It was aerated with 5 % CO_2 in air and stirred just sufficiently to prevent cell sedimentation. Further details of the culture vessel and subsequent modifications are presented in figure 1.

The information sought required comparison of cultures, or series of cultures, each illuminated at approximately the same incident irradiance but differing in terms of one defined variable. Each culture was operated under a chemostat (6) system of a constant rate of dilution with fresh medium and an equal rate of withdrawal of algal suspension. Such management provided a steady-state system similar to that which would be a method of choice in any practical large-scale culture. Unfortunately, the low specific growth rates of algae make the chemostat system sluggish in reaching a steady-state and in practice each culture had to be maintained for 7 to 12 days.

DIFFUSING CONE

One attack upon the problem of light-saturation is to find some physical method of attenuating the high irradiance of sunlight without energy loss, i.e., by spreading it out over a greater area as viewed by the cells of the culture. Of the several methods which have been suggested (3, 9) the most attractive is the use of diffusing cones held base-up and projecting into a deep culture. The culture vessel was designed to compare yields in a culture with and without a diffusing cone. In initial control experiments without use of the cone it became apparent that yield was dependent upon cell concentration. The preceding paper (4) describes yield as a function of cell concentration and therefore provides the control or base line for study of effects of adding the diffusing cone.

Our first cone was turned from a solid lucite rod, leaving a very finely turned thread to give a diffusing surface. The diameter of the base was 50 mm and the height of the conical portion was 244 mm. The cone was held in an opaque diaphragm of 50 mm diameter. The diffusing conical surface for light output had an area of 192 cm^2 as compared to a base input area of 19.6 cm^2. The intent was to achieve a 10 : 1 attenuation of input irradiance. We attempted to obtain a profile of the output irradiance over the surface of the cone but were able to do this only in a relative fashion. When immersed in water and illuminated through the base most of the light emerged from the lower third of the cone.

Since an algal suspension is itself a diffusing layer it appeared that an inverted cone might give similar results even with a clear surface. Accordingly, our second model was a hollow glass cone. It was held under an opaque diaphragm of 47 mm diameter in order to restrict the input beam to the ID of the top opening. The OD was 50 mm and the height of the conical portion was 227 mm. The light input area was 17.35 cm^2 and the output area 178 cm^2.

The arrangement of the cones in the culture chamber is shown in figure 1. By use of the cones the culture volume was decreased by about 150 ml from the original volume of 1,010 ml.

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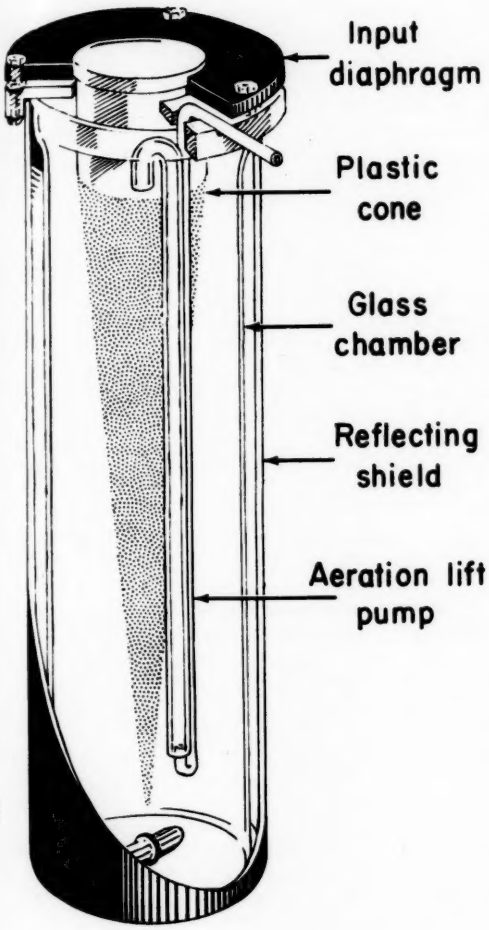

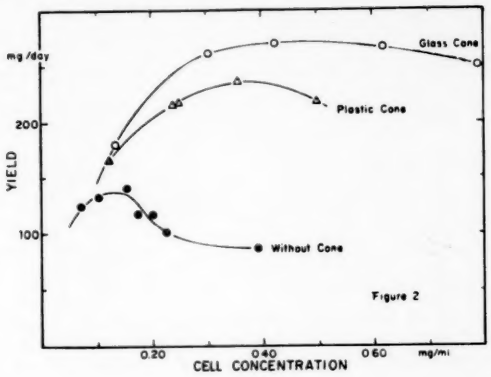

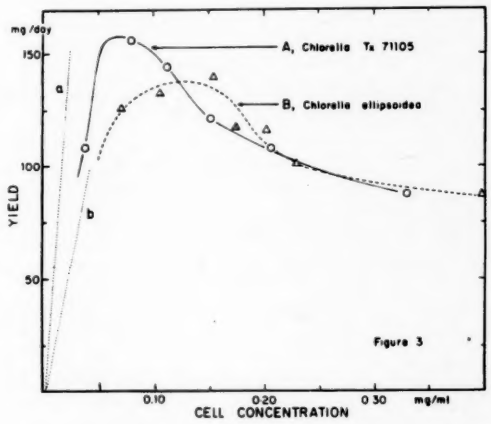

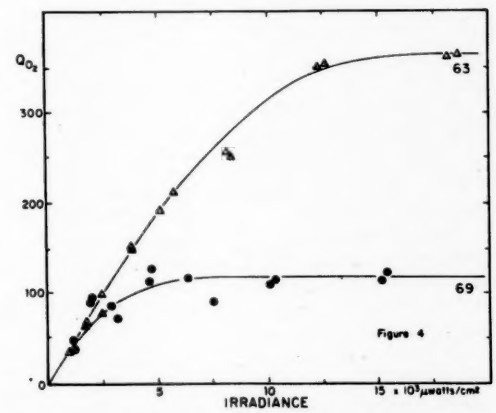

FIG. 1 (*left*). The growth chamber with plastic cone in place. The cutaway drawing shows details of the cone, the top input diaphragm, the glass chamber, and the surrounding reflecting shield. The inserted glass tubes made a lift pump delivering aerated suspension to the top of the culture with minimum splashing.

FIG. 2 (*top right*). Effects of the plastic and glass cones. The lower curve, showing performance without the cone, is taken from the preceding paper (4) with data plotted only for the last seven experiments. *Chlorella ellipsoidea* at 25° C.

FIG. 3 (*center right*). Comparison of yields without a cone for Chlorella Tx71105 at 35° C (A) and *Chlorella ellipsoidea* at 25° C (B). The dotted curves (a) and (b) represent the respective limits imposed by maximum specific growth rates taken as 6.4 days^{-1} for Chlorella Tx71105 (a) and 3.0 days^{-1} for *Chlorella ellipsoidea* (b).

FIG. 4 (*bottom right*). Rate of photosynthesis, Q_{O_2} in μl/mg-hr, vs. irradiance as measured in Warburg buffer 0.190 M $KHCO_3$ plus 0.010 M Na_2CO_3 for cells from cultures 63 and 69 of Chlorella Tx71105 at 35° C.

TABLE I

SUMMARY DATA

EXPT.	IRRADIANCE kcal/day	CELL CONC mg/l	YIELD mg/day	EFFICIENCY* %	SPECIFIC GROWTH RATE** day^{-1}	RATE RESPIRATION μl O_2/mg-hr	CHLOROPHYLL CONTENT %
Chlorella ellipsoidea at 25° C; *plastic cone; input area* 19.64 cm²; *volume* 850 ml							
51	14.5	0.122	166	6.2	1.6	11.6	2.7
52	14.2	0.239	217	8.3	1.1	7.7	4.4
55	13.8	0.251	218	8.6	1.0	5.8	3.7
54	14.0	0.357	236	9.1	0.78	3.9	4.2
53	13.8	0.494	219	8.6	0.52	...	4.8
Chlorella ellipsoidea at 25° C; *glass cone; input area* 17.35 cm²; *volume* 856 ml							
57	12.9	0.137	180	7.5	1.5	9.4	2.7
56	13.3	0.305	262	10.6	1.0	4.5	4.1
59	13.2	0.424	270	11.1	0.74	3.6	4.3
58	13.3	0.620	266	10.8	0.50	2.7	4.9
60	12.8	0.784	251	10.6	0.37	1.8	4.6
Chlorella Tx71105 *at* 35° C; *without cone; input area* 19.64 cm²; *volume* 1010 ml							
65	13.7	0.038	108	4.3	2.9	...	...
70	13.6	0.080	156	6.2	2.0	18.2	...
66	14.0	0.112	144	5.6	1.3	16.7	...
63	13.8	0.152	121	4.7	0.79	12.1	...
67	14.4	0.207	108	4.1	0.53	8.8	...
69	14.2	0.330	87	3.3	0.27	7.4	...

* Yield × 0.0054/irradiance; heat of combustion of cells produced taken as 5.4 cal/mg.
** Yield/vol. × cell concentration.

Summarized data obtained with the cones are presented in table I [cf. (4), table II] and the yields compared to those previously obtained without the cone are shown in figure 2. Two advantageous effects of the cones are clearly evident. First, maximum yield or efficiency in utilization of input light is increased about twofold. Second, the optimum cell concentration is displaced toward higher values at which harvesting becomes more economical in terms of volume of suspension to be processed.

USE OF HIGHER TEMPERATURE ALGA. All of the experiments reported above were done with *Chlorella ellipsoidea* Gerneck at 25° C, which is close to the optimum temperature. A second series of experiments was designed to make a comparative evaluation of an alga of higher temperature tolerance. For this purpose we chose Chlorella Tx71105 (8) maintained at a temperature of 35° C. Culture management in all other respects was similar to that used in the preceding report (4) and without introduction of a cone.

The results summarized in table I and figure 3 provide comparison of performance of the two strains at temperatures close to their respective optima. Chlorella Tx71105 at 35° C gave a maximum yield about 15 % higher and achieved at lower cell concentration. Auxiliary measurements of characteristics of the cells and the cultures of Chlorella Tx71105 were attempted but were less satisfactory than those previously reported for *Chlorella ellipsoidea*. Our lead sulfide cell was more difficult to use at the higher temperature and the characteristic of light penetration into the culture could not be determined satisfactorily. Chlorophyll analyses were attempted but proved unsatisfactory for this strain because of incompleteness of extraction by boiling methanol.

Irradiance curves for the harvested cells were measured in a light beam of the same spectral character used for the cultures. The single light beam available required use of the Warburg carbonate-bicarbonate buffers and repeated measurements on a single vessel at randomly ordered values of irradiance. In spite of lack of precision, which reflects known limitations in the procedure, the irradiance curves followed the same trend and demonstrated the same phenomenon previously observed with *Chlorella ellipsoidea*. Two curves presented in figure 4 were chosen from the set because of their greater reliability; each curve contains data from duplicate experiments on different days. The curve for culture 69, describing cells grown at high cell concentration and low average irradiance per cell, shows a low irradiance for light-saturation and a low maximum rate of photosynthesis.

DISCUSSION

The experimental conditions were purposely chosen to study the special or limiting case of an algal culture under continuous sunlight irradiance. Evalu-

ation in terms of practical application requires additional consideration of limitations in the experimental conditions used.

DIFFUSING CONE: The results demonstrate a twofold increase in yield and efficiency of input light utilization by a diffusing cone of arbitrarily chosen geometry. As applied to the practical case of a large culture under diurnal solar illumination there are opposing factors which would enhance or reduce the observed gain in yield. Obviously the gain will be reduced under diurnal sunlight since the most serious effects of light-saturation occur only during a portion of the day. On the other hand a large culture, having its surface covered with close-packed cones, would have little loss of light from the sides of the culture and could take more complete advantage of the cone principle. We have noted previously (4) the consequences of the unmeasured light losses which occurred from the sides of our culture vessel (edge effects). These are considered significant even though minimized by a surrounding reflecting shield. We failed in attempts to obtain a quantitative measure of these losses and can only record that they appeared considerably larger with the cone than without it.

The term "diffusing cone" is intended in a generic sense to describe a static optical device for distributing the incident surface irradiance over a larger area within a deep culture. The choice of optimum geometry has not been examined. For a large culture the use of pyramids rather than cones would allow closer packing and complete use of the surface. And it is not clear that either pyramids or cones should have straight sides for optimum effects. For attainment of maximum yield the intent is only that the incident light be so distributed that no cell of a culture is ever light-saturated. Visual inspection of our experimental cones showed that the cone surface did not radiate uniformly. Hence the desired 10:1 attenuation was not achieved and some areas of light-saturation did occur.

In short, the observed twofold gain in yield by use of the cone was obtained in spite of increased edge losses and in spite of lack of attainment of uniform light distribution. It would appear that practical test of the diffusion cone principle is merited for mass cultures under diurnal sunlight illumination. For the proposed use of algal cultures as gas exchangers in space vessels the diffusing cone principle should be an even greater advantage.

HIGH-TEMPERATURE STRAIN: The small gain in optimum yield observed with Chlorella Tx71105 at 35° C as compared with *Chlorella ellipsoidea* at 25° C is considerably less than might have been expected. Furthermore, even this small advantage was obtained only at low values of cell concentration. These findings suggest re-examination of the premises made for use of high temperature algal strains in mass culture.

In early attempts at mass culture the algae chosen were *Chlorella pyrenoidosa, Chlorella ellipsoidea,* and species of Scenedesmus, all with temperature optima at about 25° C (low-temperature strains). The first isolation by Sorokin (8) of a Chlorella with a temperature optimum as high as 39° C provided a new and important alga for mass culture. Subsequently a variety of strains of Chlorella, Scenedesmus, and other genera with temperature optima in the range of 35 to 40° (high-temperature strains) have been isolated and used in various laboratories (9). The higher temperature optima presented an important practical advantage in minimizing the requirement of cooling of cultures under sunlight illumination. Of this advantage no question can be raised. It was envisioned also that the higher rates of metabolism and growth of the high-temperature strains would allow greater yields in mass culture. The two possible bases for such an expectation should be re-examined.

As compared to the low-temperature strains, the high-temperature strains have maximum specific growth rates about threefold higher. Specific growth rate, k, is defined as $dN/N\,dt$ where N is a measure of cell quantity and t is time. For purposes of cell production in mass culture the criterion of performance is production rate or yield which for a given culture is dN/dt or kN. In general yield will be referred to some measure of culture size such as illuminated area giving dimensions such as g/m^2-day. However, for the present work the culture geometry and illumination were held constant and comparisons of yield can be made in terms of cell mass produced per day.

From experience with other microorganisms it might be reasoned that the higher maximum specific growth rates of the high-temperature strains should result in higher yield. However, the specific growth rate of an alga is governed also by the effective irradiance per cell and its maximum value is attained only at light-saturation. All the cells of a culture cannot be maintained at light-saturation without considerable transmission and loss of light. Furthermore those cells which are maintained at light-saturation cannot be working at maximum efficiency. All experimental work has confirmed the early observation of Ketchum et al (1) that "the theory of the optimum catch" applies to algal cultures, that maximum yield is attained under conditions at which the cells are growing at less than their maximum specific growth rate. Maximum yield is to be expected at a cell concentration such that most of the incident light is absorbed. It follows that yield is governed by efficiency of light utilization rather than by specific growth rate.

A second reason to anticipate advantage of the high-temperature strains under sunlight illumination lies in an expected higher point of light-saturation. For example, suppose that the point of light-saturation for the high-temperature strains lies at about 1,200 ft-c as compared to about 400 ft-c for the low-temperature strains. Then under the high illuminance of sunlight the losses attributable to light-

saturation will be lower for the high-temperature strains. The basis of this argument has been presented in the preceding paper (4).

Unfortunately the expectation of a consistently high point of light-saturation for Chlorella Tx71105 is denied by the experimental findings. The illuminance curves obtained by Sorokin (7) make it clear that cells grown at 39° C and 60 ft-c reach saturation at an illuminance about one-fourth as great as for cells grown at 400 ft-c. The data of figure 4, though incomplete and far less precise, confirm Sorokin's observations. The same effects are observable, even under an incident irradiance of full sunlight, when cell concentrations are so high as to give a low average irradiance per cell. It is now abundantly clear that a fixed irradiance curve of photosynthesis is not an intrinsic character of an algal species (2, 4, 5, 8).

The data of figure 3 show for Chlorella Tx71105 at 35° C a maximum yield only about 15 % higher than for *Chlorella ellipsoidea* at 25° C. This is a significant advantage but not one which would by itself dictate the choice of alga for mass culture. No greater advantage in yield under steady-state conditions has yet been shown for any high-temperature strain under any chosen conditions of illumination.

Summary

Yields (mg/day) of two strains of Chlorella were studied in steady-state cultures under a continuous visible irradiance equivalent to full sunlight. For *Chlorella ellipsoidea* at 25° C the maximum production rate was increased about twofold by use of plastic or glass diffusing cones designed to supply the incident energy over a greater surface at reduced irradiance. The diffusing cone principle appears to be a practicable means of circumventing the limitations of light-saturation under sunlight illumination. In cultures without the cones Chlorella Tx71105 at 35° C showed a maximum production rate only about 15 % higher than that obtained with *Chlorella ellipsoidea* at 25° C. The bases for earlier expectation of much higher production rates obtainable with high-temperature strains of algae have been re-examined.

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OXIDATION OF REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE BY MITOCHONDRIA FROM NORMAL & CROWN-GALL TISSUE CULTURES OF TOMATO[1,2]

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Respiration, both in higher plants and animals, is generally assumed to be mediated by a series of electron carriers, including the pyridine nucleotides, flavoproteins, and cytochromes. Many of the electron carriers and catalytic proteins for electron transport from reduced diphosphopyridine nucleotide (DPNH), reduced triphosphopyridine nucleotide (TPNH) or succinate to oxygen have been demonstrated in particulate preparations from potato tubers (7), wheat roots (14), pea seedlings (6, 19), avocado fruits (3), silver beet petiole (15), lupine seedlings (12), Aroid spadix (4, 5, 8, 17), and tobacco roots (18).

With mammalian mitochondria the ability of the enzymes of the particles to react with cytochrome c and DPNH in solution is dependent upon the tonicity of the suspending medium since there is a permeability barrier to these substances (13, 21). Humphreys and Conn (12) have found with lupine mitochondria that the rate of oxidation of DPNH and the effect of cytochrome c on the DPNH oxidation differ in media with and without added sucrose. Although there have been several indirect observations of this nature (10, 17) the tonicity problem has not been considered fully in many other experiments on DPNH oxidation by plant mitochondria.

The purpose of the present study was to examine spectrophotometrically the following electron transfers catalyzed by mitochondria isolated from normal and crown-gall tissue cultures of tomato: from DPNH to cytochrome c (DPNH-cytochrome c reductase activity), to 2,6-dichlorophenol indophenol (diaphorase activity) and to oxygen (DPNH oxidase activity), and from reduced cytochrome c to oxygen (cytochrome c oxidase activity). Special emphasis was put on the effect of tonicity of the reaction medium on these systems.

MATERIALS & METHODS

PREPARING MITOCHONDRIA: The tomato tissue cultures of normal and crown-gall origin were like those used in the previous experiments (23, 24). Tissue cultures originally derived from normal tissue are called normal, and those from crown-gall tissue are called crown gall. Mitochondria were prepared from 3-week old tissues as described before (23, 24). Dihydrogen potassium phosphate (KH_2PO_4) in the grinding medium was replaced by 0.02 M potassium phosphate buffer, pH 7.4, and the adjustment of pH with tris was omitted.

ENZYMATIC ASSAYS: All assays were performed with a Beckman DU spectrophotometer in cuvettes of 1 cm path at room temperature (24° C). The final volume of each reaction mixture was 3.0 ml. Each enzymatic activity was assayed at two or more mitochondrial concentrations. For convenience, the components of these mixtures are presented with the results of experiments. The initial reading was taken 30 seconds after a reaction was started, and subsequent readings were recorded at 30 second intervals for several minutes. Appropriate blanks were used to adjust the instrument for each reading.

Molar extinction coefficients used were 6.22×10^6 cm²/mole for DPNH (11), and 1.91×10^7 cm²/mole for 2,6-dichlorophenol indophenol (1). 1.91×10^7 cm²/mole was used as the difference between the coefficients of reduced and oxidized cytochrome c for the determination of reduced cytochrome c concentration (25).

RESULTS

The mitochondria from normal and crown-gall tissue cultures behaved similarly in the following enzymatic experiments. Only the results with mitochondria from crown-gall tissue cultures are presented as examples of the reactions. The comparison of activities between normal and crown-gall preparations is discussed in the latter part of this section.

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TABLE I

EFFECT OF COMPONENTS OF REACTION MEDIUM & INHIBITORS ON DPNH OXIDASE ACTIVITY OF MITOCHONDRIA FROM NORMAL TISSUE CULTURES OF TOMATO

CONDITION*	% ACTIVITY
Complete	100**
Without EDTA	98
Without $MgCl_2$	100
Without mannitol	22
Phosphate buffer only	24
With KCN	5
With Antimycin A	7
With ascorbic acid	102

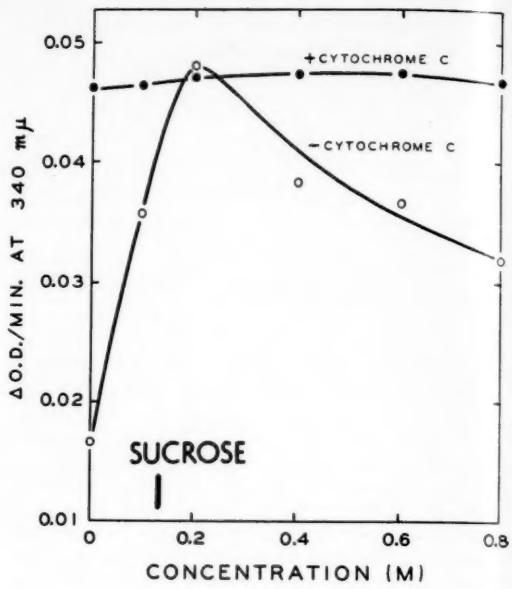
* Complete reaction mixture contained 5×10^{-5} M DPNH, 5×10^{-3} M $MgCl_2$, 5×10^{-4} M EDTA, 2×10^{-2} M phosphate buffer, pH 7.4, 0.4 M mannitol, and 0.05 ml (6 μg N) mitochondrial suspension in a total volume of 3.0 ml. KCN, 2×10^{-4} M; Antimycin A, 0.2 μg/ml; ascorbic acid, 5×10^{-4} M.

** This corresponds to 1.1 mmoles DPNH oxidized/min/mg N.

DPNH OXIDASE: The oxidation of DPNH by mitochondria was measured by the rate of decrease in optical density at 340 mμ under the conditions as described in table I. The reaction proceeded at a uniform rate for several minutes. The effect of each component of the reaction medium and inhibitors on DPNH oxidase activity is shown in table I. Omitting $MgCl_2$ or ethylenediamine tetraacetic acid (EDTA) from the reaction mixture had no appreciable effect on the rate of oxidation, whereas omitting mannitol reduced the oxidation rate to 22 %. The addition of ascorbate (5×10^{-4} M) had no effect on the oxidation. Cyanide (2×10^{-4} M) or Antimycin A (0.2 μg/ml) inhibited the DPNH oxidation by 95 % and 93 %, respectively.

EFFECT OF SUCROSE, MANNITOL, & CYTOCHROME c ON DPNH OXIDATION: The rates of DPNH oxidation at various concentrations of sucrose and mannitol with and without cytochrome c are shown in figures 1 and 2. Without added cytochrome c, the reaction rate increased with increasing amounts of sucrose or mannitol up to 0.2 M. Beyond this, the rate decreased in the sucrose mixture, whereas the maximum rate was maintained up to 0.8 M in the mannitol mixture. In the presence of cytochrome c, the oxidation rate was practically constant at the highest level at all concentrations of sucrose or mannitol.

EFFECT OF pH ON DPNH OXIDATION: The effect of pH on DPNH oxidation at the optimum concentration of sucrose (0.2 M) or mannitol (0.4 M) is shown in figure 3. Sucrose and mannitol reaction mixtures gave virtually identical curves: the rate of



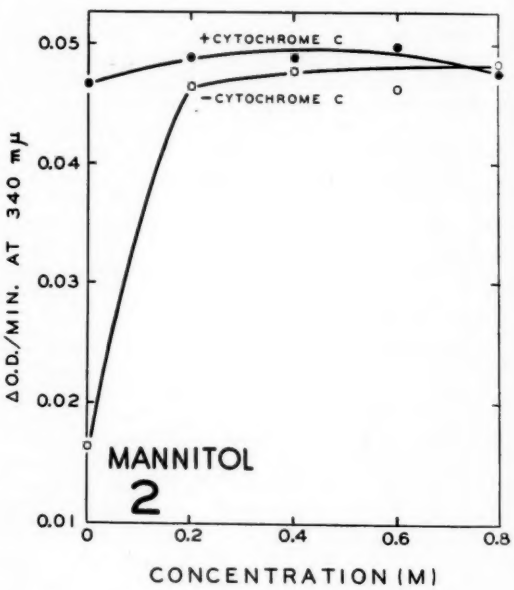


FIG. 1. DPNH oxidase activity of mitochondria from crown-gall tomato tissue cultures. Sucrose reaction mixture. Complete reaction mixtures were the same as in table I.

FIG. 2. Effect of cytochrome c (2×10^{-5} M) on DPNH oxidation by mitochondria from crown-gall tomato tissue cultures. Mannitol reaction mixture. All other components were as in figure 1. O.D./min. = change in optical density per minute.

TABLE II

EFFECT OF MANNITOL & ANTIMYCIN A ON DPNH-CYTOCHROME c REDUCTASE ACTIVITY OF MITOCHONDRIA FROM NORMAL TISSUE CULTURES OF TOMATO

ADDITION*	1ST ORDER VELOCITY CONSTANT min^{-1}
Control	0.059
With mannitol	0.018
With Antimycin A	0.015

*Control cuvette contained 5×10^{-5} M DPNH, 2×10^{-4} M KCN, 2×10^{-5} M cytochrome c, 2×10^{-2} M phosphate buffer, pH 7.4, and 0.05 ml (6 μg N) mitochondrial suspension in a total volume of 3.0 ml. Concentrations of mannitol and Antimycin A are as in table I.

oxidation was practically unchanged at the highest level between pH 6.8 and 7.7.

DPNH-CYTOCHROME c REDUCTASE: The reduction of cytochrome c was measured by following the increase in optical density at 550 mμ. Under the conditions of the test, as given in table II, the reaction showed apparent first order kinetics with respect to DPN. The reaction rate, expressed as the first order velocity constant, decreased to one-third when mannitol (0.4 M) was added to the reaction mixture (table II). Antimycin A (0.2 μg/ml) partially inhibited the reductase activity.

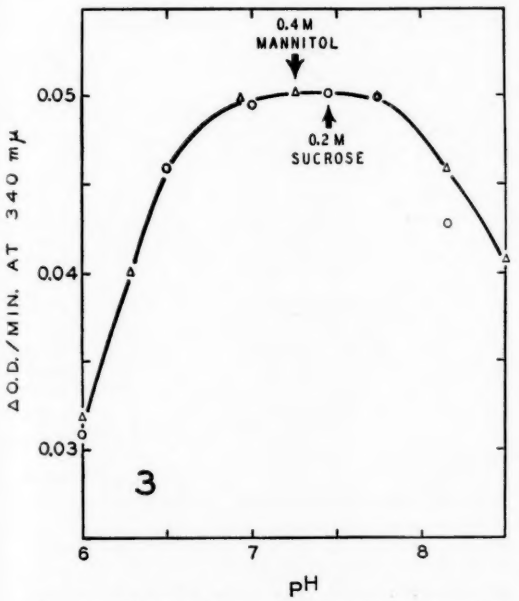


FIG. 3. Effect of pH on DPNH oxidation by mitochondria from crown-gall tomato tissue cultures. All components were as in table I.

CYTOCHROME c OXIDASE: The oxidation of reduced cytochrome c was measured by following the decrease in optical density at 550 mμ. The reaction mixture contained 3×10^{-5} M cytochrome c (approx. 80 % in reduced form), 0.2 μg/ml Antimycin A, 2×10^{-2} M phosphate buffer, pH 7.4, and 0.05 ml (6 μg N) mitochondrial suspension in a total volume of 3.0 ml. Cytochrome c was reduced in aqueous solutions (pH about 8 with tris buffer) by adding a small excess of solid sodium hyposulfite ($Na\ S_2O_4$). A fine stream of air was passed through the solution for about five minutes to oxidize excess hyposulfite.

The first order velocity constant (min^{-1}) for the disappearance of reduced cytochrome c was calculated according to Smith (20), and a value of 0.11 was obtained. The reaction rate was virtually unchanged in the presence of mannitol (0.4 M). KCN (2×10^{-4} M) blocked the oxidase activity completely.

DIAPHORASE: Diaphorase activity was measured by following the decrease in optical density at 600 mμ due to reduction of 2,6-dichlorophenol indophenol. The reaction mixture contained 5×10^{-5} M DPNH, 2×10^{-5} M 2,6-dichlorophenol indophenol, 2×10^{-2} M phosphate buffer, pH 7.4, 0.4 M mannitol, and 0.05 ml mitochondrial suspension in a total volume of 3.0 ml. Under these conditions, the reaction proceeded linearly with time for the first 2 to 3 minutes, and the rate of reaction was 2.2 mmoles DPNH oxidized/min/mg N.

COMPARISON OF RATES OF ELECTRON TRANSFER REACTIONS BETWEEN NORMAL & CROWN-GALL MITOCHONDRIA: Similar experiments as described above were carried out using mitochondria isolated from crown-gall tissue cultures of tomato. Mitochondria from crown-gall tissue cultures showed all enzymatic activities observed in mitochondria from normal tissue cultures. Their responses to the inhibitors tested and change of tonicity were also similar. However, the results of three separate, comparative experiments indicated that the rates of reactions catalyzed by crown-gall mitochondria were about 20 % lower than those by normal tissue mitochondria.

DISCUSSION

In mitochondrial oxidation of DPNH the structural integrity of the particles is important. Humphreys and Conn (12) compared the rates of DPNH oxidation by lupine mitochondria in the reaction mixtures with and without 0.5 M sucrose. In the absence of added cytochrome c, the oxidation rate was higher in the presence of sucrose than in the absence of sucrose, but in the presence of added cytochrome c the reverse was true. Adding cytochrome c enhanced the oxidation threefold in the absence of sucrose, but less than twice in the presence of sucrose.

The present experiments showed that the presence of sucrose or mannitol in the reaction mixture was essential to support the highest rate of DPNH

oxidation (figs 1 & 2). Although the maximum rates of oxidation which were attained by sucrose or mannitol were similar, the range of concentration was different. Sucrose supported the maximum rate only at 0.2 M, and above this concentration the reaction rate decreased. On the other hand, mannitol maintained a maximum rate at a wider range of concentrations, 0.2 to 0.8 M, and hence was preferable to sucrose. When mannitol (or sucrose) was omitted from the reaction mixture, or when the mitochondria were in phosphate buffer only, the rate of DPNH oxidation decreased to one-third normal. Apparently, hypotonic treatment affects the availability of cytochrome c, because an external supply of cytochrome c almost completely restored activity to the highest levels which were obtained in the presence of the optimal concentration of sucrose or mannitol.

In contrast to DPNH oxidase activity, DPNH-cytochrome c reductase activity was reduced to one-third when mannitol was added to the reaction mixture (table II), whereas cytochrome c oxidase activity was unchanged with or without mannitol. These results suggested the importance of tonicity of the reaction medium in determination of catalytic activities of mitochondria.

Oxidation of DPNH by the present mitochondrial preparations was unaffected by the addition of ascorbic acid. This response is distinct from that of the DPNH oxidation systems in soluble fractions of certain plants (2, 9, 16) and particulate preparations of wheat embryo (22).

Antimycin A inhibited DPNH-cytochrome c reductase activity of the present tissue culture mitochondria about 75%. A partial inhibition (30%) of the reductase activity has also been reported with mitochondrial particles from potato (7) and wheat roots (14). Apparently, added cytochrome c provides an alternate pathway of DPNH oxidation which can by-pass the Antimycin A-sensitive factor (7, 13). Whether the Antimycin A-insensitive pathway operates within the particles under physiological conditions is not clear.

The previous experiments (24), in which oxygen uptake was measured with an oxygen electrode apparatus, established that crown-gall mitochondria have lower oxidative activities for DPNH and Krebs cycle substrates than do mitochondria from normal tissue cells. The present experiments indicated that the ability to catalyze the electron transport reactions tested was also lower in mitochondria from crown-gall tissue cultures than in preparations from normal tissue cultures. However, there was no qualitative difference in their catalytic properties. It seems that mitochondria from normal and crown-gall tissue cultures have identical pathways for the transport of electrons from DPNH to oxygen. A possible sequence of the electron carriers is like the one suggested by Hackett (7) for the DPNH oxidation system in potato particles, and thus the plant tissue culture mitochondria show a resemblance to mitochondria of higher plants and animals.

Summary

Mitochondrial particles isolated from normal and crown-gall tomato tissue cultures were assayed spectrophotometrically for their DPNH oxidase, DPNH-cytochrome c reductase, cytochrome c oxidase, and diaphorase activities. Normal tissue particles always showed higher activities than crown-gall particles. There was no qualitative difference in their catalytic properties, and it was concluded that mitochondria from normal and crown-gall tissue cultures have virtually identical pathways for the transport of electrons from DPNH to oxygen.

The effects of the osmotic properties of reaction mixtures on these enzymatic activities, especially the DPNH oxidase system, were studied. Without added cytochrome c, the maximum rate of DPNH oxidation was obtained in 0.2 M sucrose, and in 0.2 to 0.8 M mannitol. In the presence of cytochrome c, the oxidation rate was maintained at a maximum level at all concentrations used of sucrose or mannitol. DPNH-cytochrome c reductase activity, on the other hand, was accelerated in the absence of mannitol. The activity of cytochrome c oxidase was not affected appreciably in the hypotonic solution. Ascorbic acid had no effect on the rate of DPNH oxidation by the tissue culture mitochondria.

Acknowledgments

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PIGMENT FORMATION & PHOTOSYNTHESIS IN DORMANT LETTUCE SEEDS[1]

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Dormant seeds can perform many complex metabolic processes (6, 9) and, in certain instances, mitosis and cytokinesis (5). Very little attention has been given, however, to the possibility that tissue differentiation may occur within dormant seeds. The purpose of this communication is to report an instance of tissue differentiation that we have observed in certain dormant seeds.

EXPERIMENTAL

In the course of other studies we noticed that seeds of lettuce (*Lactuca sativa* L., var. New York) became green even though germination was prevented either by preirradiation of the dry seeds with 300 to 1,000 kr Co^{60} gamma rays or by imbibition of otherwise untreated seeds in solutions of maleic hydrazide (MH). When the seeds are sown on filter paper in petri dishes containing 0.03 M MH at pH 5.7 under 30 to 100 ft-c white light at 23° C. about 60 to 70 % remain dormant. After 7 days, however, about 25 to 30 % of the dormant seeds are visibly green. Corresponding dark-imbibed controls in MH do not become green. The color is localized in an internal sector near the outer epidermis of the cotyledon which faces the light. Within this sector are chloroplasts of 4 to 5 μ diameter, indistinguishable under the microscope from the chloroplasts in the expanded cotyledons of growing seedlings.

Eighty per cent acetone extracts were prepared from green dormant seeds that had been in 0.03 M MH and exposed to 100 ft-c continuous white light for 7 days. These extracts showed the chlorophyll a peak at 663 mμ (Curve a in fig 1). This peak is absent in the extracts of dark-imbibed controls in MH

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[2] Operated by Union Carbide Corp. for the United States Atomic Energy Commission.

TABLE I

EFFECT OF LIGHT ON FIXATION OF C^{14}-BICARBONATE BY DORMANT SEEDS 7 DAYS AFTER SOWING IN 0.03 M MALEIC HYDRAZIDE*

LIGHT CONDITIONS DURING 7 DAYS IN MH*	LIGHT CONDITIONS AFTER TRANSFER TO $NaHC^{14}O_3$ SOLUTION**	C^{14} IN SOLUBLE COMPOUNDS*** AFTER 165 MIN IN $NaHC^{14}O_3$
		cts/sec per seed
Darkness	Darkness	11
Darkness	900 ft-c	11
100 ft-c	Darkness	17
100 ft-c	900 ft-c	128

* The seeds were sown on solutions of MH at pH 5.7 at 23° C (see text).
** The seeds were put with 0.4 ml of a solution of 62.5 μc/ml at pH 7 in stoppered vials of 2.1 cm diameter and 3.9 ml volume. Temperature was 25° C.
*** After 165 minutes in the C^{14}-bicarbonate, 7 seeds from each treatment were extracted (without washing), with boiling 80 % ethanol, hot and cold water. For each group of seeds the extracts were combined. An aliquot was dried under a heat lamp and assayed for radioactivity.

(Curve b in fig 1). Curve c in figure 1 shows the calculated absorption spectrum due to chlorophyll alone in the extract of seeds exposed to light. This was calculated on the assumption that the ratio of chlorophyll a to chlorophyll b is 2.9 (8). The optical density of the nonchlorophyll pigments in the extract of light-exposed seeds (Curve a minus Curve c) at 440 to 450 mμ is several times greater than that in the extract of the dark-imbibed control seeds (Curve b) at these wave lengths. Consequently the dormant seeds exposed to light also synthesized pigments other than chlorophyll. Presumably these nonchlorophyll pigments are carotenoids, since they have maximal absorption in the blue region of the visible spectrum,

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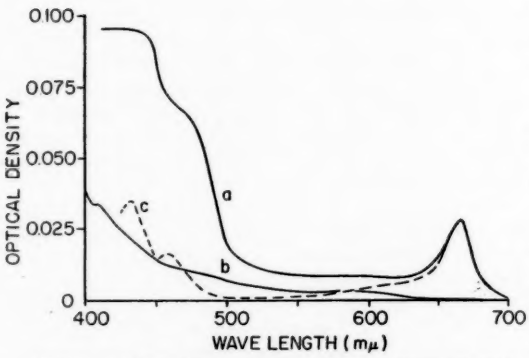

FIG. 1. Absorption spectra of 80 % acetone extracts of dormant seeds 7 days after sowing in solutions of 0.03 M maleic hydrazide at 23° C. a. Seeds exposed to 100 ft-c continuous white light after sowing. b. Seeds that had been in continuous darkness after sowing. c Calculated absorption due to chlorophyll component of Curve a (see text). In each case the optical density refers to an extract of 25 seeds made up to a volume of 5 ml.

and since carotene synthesis accompanies chlorophyll synthesis in developing chloroplasts (3).

The green dormant seeds also developed the capacity to perform photosynthesis, as shown by a light- and chlorophyll-dependent fixation of C^{14}-bicarbonate (table I) with label appearing in sugar phosphates

TABLE II

PHOTOSYNTHETIC FIXATION BY GREEN DORMANT SEEDS*

COMPOUND	% OF SOLUBLE C^{14} FIXED**
Sucrose	16
Citrulline	16
Glutamine	15
Alanine	13
Aspartic acid	11
Sugar phosphates	5
Citric acid	5
Serine	2
Glycine	1
Succinic acid	1
Malic + glyceric acids	1
Fumaric acid	0.2
Unidentified	14

* Distribution of C^{14} among compounds in the extract containing 128 cts/sec per seed in table I. The soluble compounds were separated and identified by 2-dimensional paper chromatography (using phenol-water and butanol-propionic acid-water as solvents) and the radioactivity determined by methods described by Bassham and Calvin (1).
** The percentages are calculated so that the total C^{14} on the chromatogram equals 100 %.

and sucrose (table II). Since only a part of one cotyledon became green, occurrence of a measurable amount of dark fixation would be expected even in the green seeds exposed to the tracer in the light (6). Considerable dark fixation is indicated by the low

ratio of light- to dark-fixation in the last two columns of table I. In fully green leaves at 900 ft-c this ratio is usually about several hundred (R. Rabson, personal communication).

Discussion

Lettuce seed dormancy resulting from either gamma-irradiation or MH treatment seems similar to dormancy resulting from other experimental treatments and from naturally occurring environmental conditions. In all these cases the dormancy can be broken by similar chemical agents and light (4, 7). This is not intended to suggest, however, that dormancy resulting from gamma-irradiation or MH treatment necessarily must be identical in all respects with other possible types of dormancy.

The formation of apparently normal, functional chloroplasts probably involves the synthesis of specific types of protein (2). The purpose of this communication is to point out that a failure to germinate in general cannot be caused by a complete incapacity to perform developmental processes and differentiation. This conclusion is consistent with recent studies on cell division and cell expansion in seed germination, showing that the initiation of germination can be correlated only with expansion (5). Seed dormancy must therefore be attributed to a block that specifically prevents elongation of the embryonic axis, and not to a general prevention of developmental processes and differentiation.

Summary

Lettuce seeds prevented from germinating by maleic hydrazide can develop chloroplasts in an internal sector of cotyledons exposed to light. These chloroplasts appear identical under the microscope with those normally found in expanded cotyledons of germinated seedlings. Chlorophyll and carotenoid-like pigments can be extracted from such seeds. These seeds perform a light-dependent fixation of C^{14}-bicarbonate, with radioactivity appearing in sucrose, sugar phosphates, and amino and keto acids. Similarly treated dark-imbibed control seeds do not develop chlorophyll or the capacity for photosynthesis. These findings furnish an instance of tissue differentiation in dormant seeds that is both anatomical and physiological.

Acknowledgments

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ISOLATION OF INDOLE-3-ACETIC ACID FROM CORN KERNELS & ETIOLATED CORN SEEDLINGS [1, 2]

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INTRODUCTION

Indole-3-acetic acid (IAA) has been commonly accepted as an important plant auxin (4, 14, 20). Grain of corn (*Zea mays* L.) is a rich source and milligram quantities have been isolated by alkaline hydrolysis of the mature kernels (2, 16), or by direct extraction from immature kernels (15). A number of reports have appeared concerning occurrence of IAA in vegetative portions of plants, but in many cases diethyl ether has been used as the extracting solvent in a manner which can allow the enzymatic conversion of tryptophan to IAA (40). In other cases, the existence of IAA is presumed from the bioassay of chromatograms run in a single solvent. Thus proof that free IAA is generally distributed in growing plant tissue is not yet available (1).

In a previous study (35), relatively large amounts of IAA have been detected in *Ustilago Zeae* (Beckm.) Ung. tumors and smaller amounts in healthy, early-tassel-stage corn stalks.

The objective of the present study was to isolate free IAA from vegetative plant tissue using sufficiently rigorous isolation and assay procedures to establish its identity. Methods were developed to permit the use of kilogram quantities of plant tissue, and the addition of trace amounts of IAA–2–C^{14} facilitated evaluation of losses during isolation.

METHODS

EXTRACTION, PAPER CHROMATOGRAPHY, & ELECTROPHORESIS. Michigan 350 hybrid corn was soaked in water for 4 hours and germinated on wet absorbent paper in plastic trays at 25° C in the dark and 90 % relative humidity. The 5-day-old shoots were harvested by cutting off the coleoptile plus the first internode. The diethylether used was purified just before use by shaking with a slurry of $FeSO_4$ in water and then distilling.

The plant tissue was homogenized in a blendor with sufficient 95 % ethyl alcohol to make the final concentration 80 % ethanol. After 1 to 4 hours, the ethanol homogenate was filtered and the residue re-extracted once or twice with 80 % ethanol. The combined ethanolic extracts were freed of ethanol in vacuo using a rotating evaporator and a bath temperature of 55° C, or in later experiments with a vacuum spray drier at 35 °C. In experiments where IAA–2–C^{14} was added to permit recovery calculation, it was added to the ethanol filtrate or to the homogenate prior to filtration, with identical results. At this stage one of two methods was employed for extraction of IAA into ether. These alternative methods are designated in the text as acid or alkaline extraction procedures.

For the acid extraction procedure the aqueous concentrate (500–1000 ml) was adjusted to pH 4.0 with 5 N H_2SO_4 and the resultant precipitate removed by filtration through glass wool. The filtrate was then extracted three times with diethylether, and the pooled ether extract was shaken three times with 8 % $NaHCO_3$. After acidification of the combined bicarbonate fractions to pH 4.0 with 5 N H_2SO_4, acidic substances were again extracted into ether.

For the alternative alkaline extraction procedure, the aqueous concentrate was adjusted to pH 8 with $NaHCO_3$ and extracted three times with ether. The combined ether fractions were extracted once with 8 % $NaHCO_3$ and the bicarbonate phase combined with the aqueous concentrate. After acidification to pH 4 with 5 N H_2SO_4 (& filtration) the procedure described above was used.

The ether fractions, obtained by one of the above procedures, were dried over anhydrous Na_2SO_4 at 1° C, decanted from the Na_2SO_4 and the ether removed in vacuo. The residue was washed three times with 5 ml of ether and the combined ether fractions reduced in volume to about three milliliters. This fraction was applied to a buffered ether-water partition column (see below) and eluted with ether. The IAA containing fractions were pooled and dried over anhydrous sodium sulphate at 1° C. The dry ether was evaporated and the residue was taken up in a few drops of ethanol for paper chromatography.

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Whatman 3MM or Whatman 1 paper was used for ascending chromatography using isopropanol, ammonium hydroxide, water solvent (8:1:1, v/v) in most cases. The sample was usually applied as a band with known IAA spotted at one end so as to overlap the unknown slightly. Chromatography was conducted in the dark at 1° C for 20 to 30 hours, after equilibration for 8 to 12 hours. IAA was located on the paper by a light-blue fluorescence under shortwave ultra-violet radiation (2537 A) or by spraying (with Ehrlich's reagent) a strip cut from the edge of the band containing the co-spotted IAA. The Ehrlich's reagent contained 1 g *p*-dimethylaminobenzaldehyde dissolved in 91.2 ml of 95 % ethanol and 8.8 ml of concentrated HCl. Chromatograms were eluted with 50 % ethanol and the eluant concentrated to 1 ml in vacuo. Sometimes rechromatography in the same solvent or electrophoresis was required for further purification.

Paper electrophoresis was conducted in a closed strip, solid support, plexiglass chamber using Whatman 3MM paper, 0.1 M citrate buffer, pH 5.25, 200 or 250 volts, and a temperature of 1° C. Under these conditions, the mobility was 2.94×10^{-5} cm²/v-sec.

In some instances, continuous flow electrophoresis proved to be valuable. The continuous flow electrophoresis unit was that of Durrum (10). Whatman 3MM paper was washed successively with ethylenediaminetetraacetate at pH 8.0, 0.1 N HCl, and distilled water. Electrophoresis at pH 6.55 with 0.025 M phosphate buffer at room temperature (in the dark) was found to be satisfactory. Under these conditions, the IAA migrated half way toward the positive electrode when the wick was 1 inch from the negative edge, while the pigments did not migrate.

Usually, 4 ml of Salkowski reagent (32) were added to 1 ml of eluted concentrate for the detection of IAA. The red color complex was measured with a colorimeter using a wide band, 540 mμ filter. Standard concentrations of IAA were included in each determination. The modified Avena section straight growth test of McRae et al (23) was used for bioassays.

The IAA-2-C^{14} used in these studies had a specific activity of 9.18×10^4 d/m/μg (31). Due to possibility of radio-decomposition, the sample was purified by column chromatography and paper chromatography twice during the course of these investigations. The stock solution was kept as a very dilute solution in ethanol and stored at −20° C. After the initial purification, no decomposition products were noted when the IAA-2-C^{14} was chromatographed.

COLUMN SEPARATION. Corn seedlings contain high concentrations of yellow-brown phenol-like pigments.[4] Chromatograms of corn seedling extracts were colored and it was impossible to detect μg amounts of IAA by fluorescence or with Ehrlich and Salkowski reagents. Column chromatography has been used in the isolation of IAA (11, 21, 22, 24). However, none of those columns appeared to be entirely satisfactory under our particular conditions.

The column chosen for use was a true partition column employing a buffered aqueous phase adsorbed on analytical grade diatomaceous silica (Celite) using ether as the mobile phase. This partition was similar to that used by Holley et al (18) except these workers used a Craig apparatus. Analytical grade Celite was thoroughly ground in a mortar with 0.8 times its weight of phosphate buffer. Portions of the damp Celite were slurried in the column with buffer saturated ether and the slurry packed with a glass rod. A flow rate of 0.5 to 2.0 ml per minute was obtained with 3 to 5 pounds pressure per square inch of nitrogen. The column was eluted with ether saturated

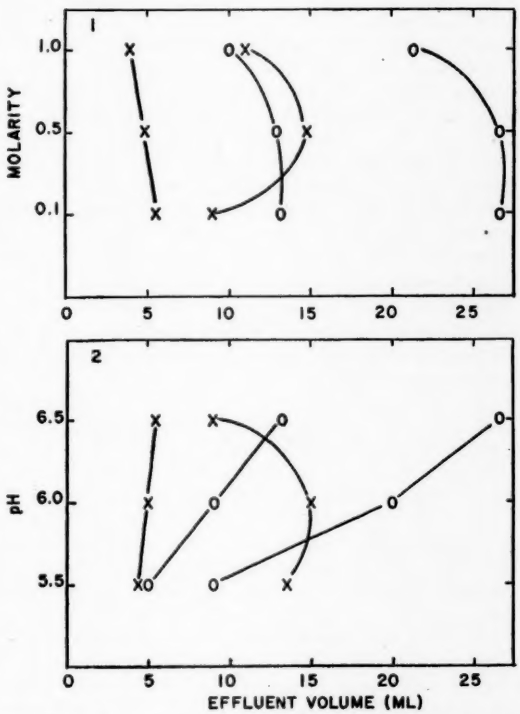

FIG. 1 (*top*). The effect of molarity of the potassium phosphate buffer (pH 6.5) on the migration of IAA and corn shoot pigments on a 10 g Celite column 25 mm (O.D.) in diameter. The boundary lines indicate qualitatively where elution of IAA (O–O), and pigments (×–×) begins and ends.

FIG. 2 (*bottom*). The effect of pH on the migration of IAA and corn shoot pigments on a 10 g Celite column 25 mm (O.D.) in diameter. The boundary lines indicate qualitatively where elution of IAA (O–O), and pigments (×–×) begins and ends.

[4] These are breakdown products of the cyclic hydroxamate 1,3-dihydroxy-2-keto-6-methoxybenzoxazine (17, 38).

with the same buffer used on the column. Figures 1 and 2 indicate the effect of molarity and pH of the phosphate buffer on the elution of IAA from a 2.5 cm column containing 10 g of Celite. High buffer concentrations depressed the solubility of IAA in the aqueous phase. Over the small range of hydrogen ion concentrations tested, there was an almost linear relationship between pH and rate of movement of IAA through the column.

Maximum separation of IAA and the corn pigments was obtained with 0.1 M phosphate buffer at pH 6.5[5]. Under these conditions, with a 1.8 cm diameter, 20 g column, IAA came off in the 40 to 60 ml fractions. (The IAA containing fractions from a 2.5 cm, 20 g column or a 5 cm, 100 g column were, respectively, 50–70 ml & 200–500 ml.) The highest concentration of IAA was found just after the first tube or two indicating that the IAA band trailed upward on the column. Usually 2 to 3 ml of ether were collected per tube, and the IAA located by adding 4 ml of Salkowski reagent and shaking. The color development was somewhat slower than in the regular Salkowski assay.

Results

Isolation & Recovery Experiments Employing Unlabeled Carrier IAA. One hundred μg of IAA were added to 1 liter of 95 % ethanol and carried through concentration, ether extraction, column and paper chromatography. The IAA was located on the paper chromatogram by UV fluorescence and eluted with 50 % ethanol. The ethanol was removed in vacuo at 55° C, 2 ml of water were added, and the amount of IAA estimated by Salkowski assay. In two experiments, recoveries were 50 and 56 %. Other similar experiments, where losses for individual steps were evaluated, indicated small cumulative losses for each operation.

Eight experiments without added IAA, using the acid ether extraction procedure, were conducted using from 178 to 1,000 g of 5-day-old etiolated corn shoots. Since no IAA was detected, recovery experiments were necessary. In the first experiment, 100 μg of IAA were added to the 80 % ethanol extract of 1,000 g of corn shoots. Another 100 μg of IAA were mixed with the residue and the residue re-washed with 2 liters of 80 % ethanol. Recovery was 25 % in both cases. In the second experiment, 120 μg of IAA added to 316 g of 5-day-old corn shoots before homogenization resulted in 27 % recovery. In the third experiment, 200 μg of IAA added to 700 g of corn shoots resulted in 30 % recovery. It appeared that recovery for the overall isolation procedure using 100 to 200 μg IAA per kilogram of corn shoots is 25 to 30 %.

One attempt was made to isolate IAA from 11.3 kilogram of 5-day-old etiolated corn shoots using the acid ether extraction procedure. Five lots of corn shoots were grown over a 30-day interval. Each individual lot was harvested, extracted with 80 % ethanol, and worked up to the partition column stage. This acid ether fraction was concentrated to dryness in vacuo and stored at −20° C. The pooled lots were dissolved in ether and the ether concentrate divided into three portions. Each portion was partitioned on a 5 cm diameter, 100 g column, and the IAA fractions pooled. The pooled eluant was electrophoresed in two runs using the continuous flow unit. No IAA could be detected by Salkowski assay. When 26 μg of IAA were placed on the 5 cm diameter partition column, recovery was 77 %; and when 10 μg of IAA were run on continuous flow electrophoresis, recovery was 70 %.

Isolation Experiments Using IAA–2–C^{14}. The above recovery experiments might not be valid owing to the addition of relatively large amounts of IAA. Therefore, low level experiments with corn shoots, using IAA–2–C^{14}, were carried out. The use of trace amounts of high activity IAA–2–C^{14} was valuable in several ways. Recovery data thus could be obtained during the actual isolation experiments. Even if losses were 90 % during isolation, addition of 1 to 3 μg of radioactive IAA was sufficient for recovery evaluation. The results of these isolation experiments are summarized in table I.

In experiment 1, no C^{14}-labeled IAA was recovered for unknown reasons. In experiments 2 through 5, employing the acid extraction procedure, recoveries of C^{14}-IAA averaged 14 %. About two μg of IAA could have been detected and accurately measured by the Salkowski assay procedure used. Thus one may calculate upper limits for the amount of free IAA which could have been present and escaped detection. For experiment 2, this would be 2 μg/0.145 minus the 1.89 μg IAA–C^{14} added or 11.9 μg IAA/Kg of fresh tissue. Since no trace of color was observed, one must conclude that substantially less than this amount of free IAA exists in corn as estimated by the acid

Table I

Recovery of IAA–2–C^{14} From Etiolated Corn Shoots & Detection of IAA in Corn Shoots[1]

	Fr wt	μg IAA–2–C^{14} added	% Recovery of IAA–2–C^{14}	μg IAA/kg by Salkowski assay corrected for recovery
1.	1,000	1.89	0	0
2.	1,000	1.89	14.5	0
3.	1,000	2.84	12.4	0
4.	1,000	1.89	12.0	0
5.	1,000	2.84	16.1	0
6.	3,000	1.65	28.1	13.3

[1] In experiments 1 through 5 the acid extraction procedure, and in experiment 6 the alkaline extraction procedure, was employed.

[5] The partition column sometimes failed when alkaline extraction was used.

extraction procedure. The comparable values for experiments 3 to 5 would be 13.2, 14.7, and 9.6 μg/kg. In experiment 6 where the preliminary alkaline extraction procedure was employed, an IAA concentration corresponding to 13.3 μg/kg was found.

FREE & BOUND IAA IN CORN GRAIN. Corn grain was a rich source of free and bound IAA in confirmation of numerous reports of the occurrence of IAA in the grain (2, 7, 15, 29, 30, 37, 41). The soaked grain was allowed to germinate in the dark for 0, 12, or 24 hours. The samples were ground and then extracted three times with 80 % ethanol. A gummy, yellow-orange IAA liberating precipitate was obtained upon concentration of the ethanol extracts. Following washing with water, the precipitate was dissolved in 150 ml of 1 N KOH and heated 1 hour at 100° C. Upon acidification, the re-precipitated material was removed by filtration through glass wool. The filtrate was carried through the IAA isolation procedure and the residue was again heated with alkali.

The free IAA and IAA released by hydrolysis (table II) were characterized by electrophoresis, and by paper chromatography in four solvents. The estimate of concentration, obtained by the Salkowski assay, was verified by direct spectrophotometric assay of chromatograms sprayed with Ehrlich's reagent and by Avena section bioassay.

It has been suggested (12, 27, 37), that IAA may arise from proteins containing tryptophan. Though zein is low in tryptophan, it is the major alcohol soluble protein. As reported (3), alkaline treatment of zein yielded no IAA and similar alkaline treatment of 100 mg of tryptophan yielded no IAA. To further determine whether or not the precursor was protein, about 500 g of corn meal was extracted three times with 1 liter portions of 0.01 M $MgCl_2$. The combined extracts were cooled to 1° C and the protein precipitated by saturation with ammonium sulfate. The protein was collected by filtration and washed with 80 % ethanol. The ethanol was removed in vacuo at 60° C. The ethanol-soluble and ethanol-insoluble proteins were heated for 1 hour in 1 N KOH at 100° C. IAA was isolated from only the alkaline treated ethanol-soluble protein.

Sugar beet leaves and roots, green peas, cucumbers, potatoes, bananas, and etiolated pea shoots were examined qualitatively for the presence of IAA and other Ehrlich reactive substances by the described methods. Vegetative sugar beet roots and leaves and possibly pea shoots contained IAA in sufficient quantities to be detected.

TABLE II

ISOLATION OF FREE & BOUND IAA FROM CORN GRAIN [1]

EXPT.	μg OF IAA/kg		
	FREE	1ST HYDROLYSATE [2]	2ND HYDROLYSATE [2]
1	82	186	98
2	115	509	63
3	171	...	...
4	330	...	...
5[3]	16,800	...	...

[1] In experiments 1 to 4, Michigan 350 grain was soaked for 4 hours. In experiment 1, it was ground after soaking; in 2 and 3, it was germinated 12 hours at 25° C, and in 4, germinated 24 hours at 25° C.

[2] IAA obtained following hydrolysis of the 80 % ethanol-soluble, water-insoluble residue for two successive 1 hour periods with 1 N KOH at 100° C.

[3] Market stage yellow sweet corn kernels purchased locally.

DISCUSSION

Housley, Booth, and Phillips (19) reported failure to find IAA in corn seedlings, and our preliminary studies confirmed this finding. Reinert and Forstman (25) obtained similar results, and postulated some sort of binding of IAA in a manner perhaps similar to that investigated by Tegethoff (33) in corn scutellum. In the present case using acid extraction, recovery of 1 to 3 μg of IAA was only the order of 12 to 14 %; while recovery of 100 μg was about 25 %. The increased recovery when the aqueous concentrate was made alkaline may suggest some reversible binding. It is doubtful if actual destruction of IAA at pH 4.0 is a factor. Brian (5) has found binding of 2-methyl-4-chlorophenoxy acetic acid is greater at low pH and that crude corn extracts bind comparatively large amounts. One may question whether or not any free IAA exists in vivo. From the present work, the occurrence of *appreciable* free IAA in ethanolic extracts of corn shoots must be considered doubtful, since added IAA (1–3 μg) is largely lost. One could conclude that endogenous and exogenous free IAA is bound to some ethanol soluble component upon grinding the tissue. An alternative is that all the IAA in vivo occurs in the form of a very labile complex. The binding of exogenous IAA may or may not be related to this complex. Adjustment of the pH of the aqueous concentrate to 8.0 for preliminary removal of neutral ether soluble substances, led to an estimate of 13.3 μg of IAA per kilogram. IAA should have been detected, at least qualitatively, in the experiments using acid ether extraction. Since this was not the case, either endogenous IAA was liberated from a complex by alkaline pH adjustment or was produced from tryptophan or other precursors.

In the present work, IAA could actually be detected in corn shoots or roots by wet ether extraction for 3 hours at 4° C. Furthermore, subsequent 70 % ethanol extraction of the residue resulted in detection of IAA. However, no IAA could be detected in ethanol or subsequent ether extracts when the initial extraction was with 80 % ethanol. The greater ease with which IAA is detected by wet ether extraction may be due to its ability to dissociate bound IAA. In this respect, it is interesting to note that ether can prevent polar auxin transport (36). However, Wildman and Muir (40) have demonstrated IAA produc-

tion from trytophan during wet ether extraction with a low temperature optimum.

Though IAA has been detected in Avena coleoptiles (28, 34) experiments with radioactive IAA indicate no redistribution under geo- or phototropic stimulation (6, 8, 13, 26). A complex such as here discussed might explain the experiments on tropisms demonstrating a lack of redistribution of radioactive IAA. Thus exogenous IAA might not be in equilibrium with the endogenous IAA complex.

IAA can be derived from tryptophan in vitro by preparations from various tissues (9, 14, 30, 39, 41) and production of IAA from tryptophan by a particulate fraction from corn seedlings has been observed (17). Tryptophan–2–C^{14} is converted directly to carboxyl labeled IAA (17) and the other acid ether soluble products were similar to those reported by Dannenburg and Liverman (9). However, in contrast, little labeled IAA could be isolated when excised corn shoots were fed tryptophan–2–C^{14}.

In contrast to the growing shoot, corn kernels are a rich source of free IAA (2, 7, 15, 29, 30, 37, 41) and an IAA releasing fraction, presumably identical to that of Berger and Avery (2, 3). This substance might be a protein or polypeptide since it is precipitated with ammonium sulfate. However, Stehsel (29) found the alkali labile IAA complex in immature sweet corn seed was dialyzable.

Summary

Methods found useful in the isolation of IAA from kilogram quantities of plant tissue are described. A buffered ether-water partition column technique was developed. The use of trace amounts of IAA–2–C^{14} was especially valuable during the isolation, and in evaluating the recovery. IAA was detected in 80 % ethanol extracts of corn kernels and shoots, as well as vegetative sugar beet roots and leaves. The difficulties of detection of IAA in corn shoots are discussed in detail and it is suggested IAA may occur as a labile complex in ethanol extracts of this tissue. An 80 % ethanol-soluble, aqueous-insoluble fraction from corn kernels was found to release IAA upon alkaline hydrolysis. It appears this fraction may be protein in nature.

Acknowledgments

We should like to take this opportunity to thank Dr. S. A. Gordon who supplied the IAA–2–C^{14} and Dr. H. Sell for the use of the vacuum spray dry apparatus. It is a pleasure to acknowledge the assistance of Dr. H. Fukui in the ether extraction experiments.

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NEWS & NOTES

REPORT ON 1961 MEETING OF SOUTHERN SECTION OF AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS. On February 6 to 8 the Southern Section of the American Society of Plant Physiologists met with the Association of Southern Agricultural Workers at Jackson, Miss. All the sessions were well attended. The program included 28 contributed papers and a symposium on photosynthesis.

The symposium was chaired by Dr. Hans Gaffron, Florida State University. Papers were presented by Dr. André Jagendorf, McCollum-Pratt Institute, Johns Hopkins University; by Dr. Birgit Vennesland, University of Chicago, and by Dr. Roderick K. Clayton, Biology Division, Oak Ridge National Laboratory. The symposium will be printed and copies will be available from Secretary-treasurer Joseph C. O'Kelley, Biology Dept., University of Alabama, Tuscaloosa, at the cost of $1.00.

The Annual Plant Physiologists' Breakfast was held on the morning of February 8. A first prize award of $25.00 for the best paper given by a graduate student was awarded to Mr. Theodore Holmsen, University of Florida, for a paper, "Geotropism in *Zea mays*". Another award of a copy of the Annual Review of Plant Physiology, volume 11, 1960, was made to Mr. Coleman Ward, Virginia Polytechnic Institute, for his paper, "Effects of Potassium Levels on Orchard Grass and Red Clover in Sand Culture". The annual breakfast address was given by Dr. Harold Evans, North Carolina State College, on "The Essentiality of Cobalt for Leguminous Plants Grown Under Symbiotic Conditions".

Officers elected for 1961 were chairman, Dr. Howard E. Joham, Texas Agricultural Experiment Station; vice-chairman, Dr. Robert D. Powell, University of Florida; secretary-treasurer, Dr. Joseph C. O'Kelley, University of Alabama. Elected members of the Executive Committee are Dr. Howard Teas, University of Puerto Rico; Dr. Wayne C. Hall, Texas Agricultural Experiment Station, and Dr. Robert Burns, U.S.D.A., Experiment, Georgia. Dr. Teas is the Southern Section representative to the American Society of Plant Physiologists.

—JOSEPH C. O'KELLEY, *Secretary-Treasurer*, Southern Section.

INFLUENCE OF 2,3,5-TRIIODOBENZOIC ACID, INDOLE-3-ACETIC ACID & METHOD OF SAMPLE COLLECTION ON TRANSLOCATION OF FOLIAR APPLIED RADIOCALCIUM [1, 2]

G. A. TAYLOR, J. N. MOORE, & W. O. DRINKWATER

The relative immobility of foliar-applied calcium has been demonstrated by numerous workers, and pertinent literature has been cited by Biddulph, Cory, and Biddulph (*2*). This explains the difficulty in controlling physiological disorders involving calcium nutrition in commercial practice by means of foliar applications of soluble calcium salts. Therefore, any substance promoting translocation of foliar-applied calcium would be of practical as well as fundamental interest.

Bukovac, Wittwer, and Tukey (*3*) imply that polarity may be responsible for the lack of basipetal transport of calcium in the bean plant. When polarity was suspended by anesthetization with diethyl ether, as shown by Went and Thimann (*8*), Bukovac et al found Ca^{45} was readily transported from the treated leaf. Subsequent work by Biddulph, Cory, and Biddulph (*2*) also demonstrated the pronounced positive effect of diethyl ether on Ca^{45} translocation. However, the latter workers have shown that diethyl ether did not induce calcium movement in the phloem but rather brought about transport via the xylem. Thus injury, rather than any alteration of polarity, may have been responsible for the reported effect of diethyl ether.

Kessler and Moscicki (*5*) have presented data which showed that triiodobenzoic acid (TIBA) brought about the basipetal translocation of foliar-applied calcium in the apple and tomato. They theorized that TIBA influenced Ca^{45} translocation by suspending the polar movement of auxin. Biddulph et al (*2*) reported no enhancement of calcium transport in the bean plant following treatment with TIBA when samples were obtained directly from fresh plants for radioactive assay. On the other hand, if identically treated plants were dried intact before sampling, there was considerable movement of radioactive calcium from the site of application. These workers concluded that TIBA caused injury to the plants which resulted in the movement of calcium in the xylem during the dehydration of the intact plant. Bar-Akiva and Hewith (*1*) were likewise unable to confirm Kessler and Moscicki's results using TIBA and Fe^{59} on chlorotic lemon trees.

In view of the importance of this question, further information on the auxin hypothesis proposed by Kessler and Moscicki was considered necessary. Since the manner of handling plant material is of considerable importance, as shown by Biddulph et al, the problem was also considered from this standpoint. The work reported here was carried out in two phases: A, an initial preliminary experiment to establish the methodology to be employed in subsequent work and to provide limited information regarding the factors under test, and B, the replicated main experiment from which more critical evidence was obtained.

Materials & Methods

Preliminary Experiment. Uniform plants of *Lycopersicon esculentum* Mill. var. Fireball were selected from a large population for treatment when the first flowers on the first cluster had reached anthesis. To increase uniformity and reduce the possibility of soil-borne Sr^{90} contamination the plants were grown in pure quartz sand supplied with Hoagland's no. 2 nutrient solution. One group of plants was sprayed to incipient runoff with a 100 ppm solution of TIBA containing 0.05 % Tween 20 as a surfactant. Control plants were sprayed with distilled water containing the surfactant. The foliage was permitted to air-dry, at which time the radioactive calcium solution was applied to the basal leaflet of the sixth leaf, counting from the base, by means of a milk dilution loop. The $Ca^{45}Cl_2$ solution, which had a specific activity of 0.88 millicuries per milliliter, had previously been adjusted to a pH of approximately 3.5.

The following treatments were employed in all possible combinations: A, 24- and 48-hour isotope migration period; B, 48 and 96 microcuries of Ca^{45} per plant; C, plant tissue samples cut from intact plants and identical samples obtained from plants cut at the sand level and dried 24 hours in a forced draft oven at 80° C.

Following the migration period, or after plant dehydration, four tissue samples were obtained from each plant. These samples consisted of stem and petiole sections approximately 1.5 cm in length from the following locations on the plant: A, mid-portion of the internode between the seventh leaf and the first flower cluster; B, petiole of the sixth leaf adjacent to the main stem; C, mid-portion of the inter-

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[2] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers, State University, Department of Horticulture, New Brunswick.

node between the fourth and fifth leaves and D, midportion of the internode between the second and third leaves. The dry weight of each individual section ranged from 0.04 to 0.08 gram.

Each section was ashed at 600° C in 5-ml pyrex beakers. The resulting ash was taken up in dilute HCl and transferred into 2-inch stainless steel planchets, which were then dried under infra-red lamps. A Nuclear Measurements Corp. Model PCC–10A gas flow internal proportional counter having an over-all efficiency of 40 % was employed for radioactive assay of the samples. This type of equipment was selected because it possesses a higher, more constantly reproducible efficiency for measurements of weak energy beta radiations than end-window Geiger-Müller counters (7). Frequent background counts were taken to make certain the chamber had not become contaminated from highly radioactive samples.

Main Experiment. This experiment was designed to provide additional information regarding the influence of TIBA and manner of sample collection on the translocation of foliar-applied Ca^{45}. In addition, data reflecting the influence of a native auxin, indole-3-acetic acid (IAA), on calcium translocation were desired to provide evidence for or against the concept proposed by Kessler and Moscicki. Thus, three treatments consisting of foliar applications of 100 ppm TIBA, 100 ppm IAA, and a distilled water control were each applied to two sets of twelve plants in the manner previously described. Forty-eight microcuries of $Ca^{45}Cl_2$ solution were applied to the first leaflet of the sixth leaf with a milk dilution loop.

Following a 24-hour migration period, one set of twelve plants was sampled by cutting four sections from each plant. These plant samples, subsequently referred to as "dismembered prior to drying", were taken from the same plant positions and in the same manner as those of the preliminary experiment. At the same time plants of the second group were cut off just above the sand, dried for 24 hours in a forced draft oven, and then sampled in the same manner as the first group. This latter group is referred to as "intact plants dried then dismembered". The entire experiment was replicated four times.

Since some difficulty was encountered in obtaining significant counts above background with the samples from the preliminary experiment, larger stem samples, ranging from 0.10 to 0.16 gram, were used in this experiment. Ashing and counting were carried out in the same manner as described in the preliminary experiment. After correction for background, the results were expressed as counts per minute per gram dry weight. These data were transformed to common logarithms and subjected to an analysis of variance as suggested by Goulden (4). The log transformation was employed because of the wide difference in numerical magnitude between samples dismembered prior to drying and those from plants which were dried intact prior to sampling.

Concurrently, an additional series of plants was sprayed with a 5 % suspension of calcium gluconate to determine if the foliar concentration of calcium would influence the mobility of foliar-applied Ca^{45}. The same experimental conditions used in the main experiment were imposed, except that plant samples were obtained only from plants prior to drying.

Results & Discussion

Preliminary Experiment. Radiation was readily detected in all plant samples taken from the petiole of the sixth leaf, the leaf to which the radio Ca was applied. However, with the exception of those plants dried intact prior to collection of samples, it was impossible to obtain significant counts above background from samples taken from internodes above or below the site of radiocalcium application. When plants were dried intact, counts in excess of 2,000 per minute per gram dry weight were obtained at the second and fourth internodes. This was true for control plants as well as for TIBA-treated plants. There was no difference between the other experimental variables consisting of 96 microcuries of Ca^{45}

Table I

Influence of Sampling Technique & Growth Regulating Substance on Translocation of Foliar-Applied Ca^{45}

Method of sampling	Chemical treatment	Counts/min/g dry wt Plant position*			
		A	B	C	D
Dismembered prior to drying	TIBA	960**	1,489	431	442
	IAA	1,840	2,118	2,685	1,726
	Control	1,394	1,524	920	1,093
Intact plant dried then dismembered	TIBA	133,636	154,432	27,926	18,242
	IAA	4,298	593,355	49,134	20,046
	Control	60,707	796,371	191,048	101,532

* Plant position: A, Internode seventh leaf to flower cluster. B, Petiole of sixth leaf adjacent to main stem. C. Internode fourth and fifth leaf. D, Internode second and third leaf.
** Each value represents the mean of four replications.

per plant compared with 48 microcuries per plant, and a 24-hour migration period compared with a 48-hour migration period.

MAIN EXPERIMENT. Inspection of the treatment means shown in table I reveals a good agreement with results from the preliminary experiment. Consistently high counts were obtained from all samples taken from plants which were dried 24 hours prior to sampling.

A statistical interpretation of the main effects of the chemical treatment means may be made from the data shown in table II. Pretreatment of plants with TIBA did not enhance translocation of foliar-applied calcium when all four sample positions were averaged. There was, in fact, a tendency for TIBA to reduce over-all translocation when compared to IAA treated plants and to the controls. This difference is significant only at odds of 19:1, but it is in agreement with the trend reported by Biddulph et al. Since the chemical treatment by plant position by sample preparation interaction was not statistically significant, the use of mean plant values to interpret the over-all effect of the chemical treatments on translocation is justified. The first order interaction between plant position and chemical treatments was likewise not significant.

TABLE II

EFFECT OF CHEMICAL TREATMENT ON TRANSLOCATION OF FOLIAR-APPLIED Ca^{45}

CHEMICAL TREATMENT	Counts/min/g dry wt*	MEAN LOG_{10}
TIBA	42,195	2.808
IAA	84,400	3.402
Control	144,324	3.607
	LSD 0.05 =	0.572

* Means of four replications, four plant positions, and two sampling procedures.

These data are not in agreement with those published by Kessler and Moscicki (5), who reported a positive effect of TIBA on Ca^{45} translocation in the tomato. The failure of IAA to inhibit Ca^{45} translocation, as shown in table II, is further evidence against their auxin concept. The results reported here are, however, in agreement with those of Biddulph et al in that TIBA did not influence Ca^{45} translocation.

As mentioned earlier, the most striking difference obtained in this experiment was the profound effect of sample preparation technique on apparent translocation. Samples taken from plants which were dried intact prior to sampling were a hundredfold more radioactive than similar samples cut from fresh plants and subsequently dried. (table III)

The movement of Ca^{45} in plants pretreated with TIBA during dehydration is in complete agreement with the results reported by Biddulph et al (2). In view of the data obtained here, however, it is necessary to draw a different conclusion from that of the previously mentioned workers. Biddulph et al concluded that TIBA caused injury which "induced calcium movement in the xylem during dehydration of the plant". The data reported here show that Ca^{45} mobility was as great in control plants as in those treated with TIBA (tables I & II). Thus, dehydration in itself was responsible for the movement of calcium in the plants which was independent of chemical treatment.

The disparity between the results reported here and those of Biddulph et al may lie in the fact that the latter workers did not include a control treatment wherein bean plants were dried intact prior to sampling. They do state in their paper that "repeated tests have shown that no artifacts in calcium distribution occur when intact plants were quickly dried between blotters in a forced draft oven at 85° C". It is also possible that the disparity can be attributed to differences in drying conditions or species of plant material studied. In view of the foregoing data, which clearly shows that calcium will readily become redistributed into other plant parts during dehydration, it is imperative that the possible danger of artifact occurrence in radioautography of intact plants be given serious consideration.

Pallas and Crafts (6) showed that artifacts of 2,4-D movement result when frozen intact plants are dried between warm blotters. These workers postulate that "when green plants are killed all cells are rendered permeable. The tracer, in solution in the original solvent or in the sap of cells that it has permeated, is free to move along any hydrostatic gradients that are set up during drying". Their interpretation of the nature of the mechanism involved may be applicable here.

On the other hand, since Ca^{45} is a relatively weak beta emitter, it is possible that translocation of Ca^{45} during the drying process may not be detectable in stem tissue by conventional radioautographic technique. Because of the large mass of tissue present, self-absorption might well prevent the development

TABLE III

EFFECT OF SAMPLING METHOD ON TRANSLOCATION OF FOLIAR-APPLIED Ca^{45}

METHOD OF SAMPLING	Counts/min/g dry wt*	MEAN LOG_{10}
Dismembered prior to drying	1,385	2.366
Plant dried then dismembered	179,227	4.179
	LSD 0.001 =	0.782

* Means of four replications, four plant positions, and three chemical treatments.

TABLE IV

EFFECT OF PRETREATMENT WITH CALCIUM GLUCONATE ON ABSORPTION & TRANSLOCATION OF FOLIAR-APPLIED Ca^{45}

CHEMICAL TREATMENT	PLANT POSITION*				MEAN
	A	B	C	D	
	(counts/min/g dry wt)				
5% Ca gluconate	384	330	218	858	448**
Control	1,394	1,524	920	1,093	1,233

* Plant position: A, Internode seventh leaf to flower cluster. B, Petiole of sixth leaf adjacent to main stem. C, Internode fourth and fifth leaf. D, Internode second and third leaf.
** Means not significantly different ($P = 0.05$)

of clearly defined radioautograms. The answer to this question must await future work.

Attempts to influence the mobility of foliar-applied Ca^{45} by pretreatment with calcium gluconate were not successful. The mean radioactive counts shown in table IV do not differ significantly. Translocation of foliar-applied calcium was apparently independent of the total calcium concentration on the leaf surfaces.

SUMMARY

Two experiments were conducted to determine the influence of triiodobenzoic acid and indole acetic acid on the translocation of foliar-applied radiocalcium in the tomato. Conducting tissue samples were selected from three internodes and from the petiole of the leaf used for isotope application. Two methods of obtaining plant samples were employed. In one series of plants the samples were cut from fresh intact plants 24 hours after isotope application, whereas in the second series the plants were dehydrated in a forced draft oven for 24 hours prior to sample selection.

The pretreatment of plants with triiodobenzoic acid and indole acetic acid did not have any appreciable effect on Ca^{45} translocation. Samples from plants treated with triiodobenzoic did exhibit somewhat less radioactivity than samples from plants treated with indole acetic acid or control plants.

Dehydration of intact plants in the forced draft oven resulted in an over-all increase in radioactivity of a hundredfold when compared to plants which were dismembered prior to drying. This was true regardless of chemical treatment.

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MEDIATION OF GEOTROPIC RESPONSE BY LATERAL TRANSPORT OF AUXIN[1,2]

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A suggestion concerning the mechanism of geotropic response was offered independently by Cholodny in 1924 (12) and by Went in 1926 (27). It was known by this time that a growth promoting hormone (an auxin) is produced in tips of coleoptiles and seedlings and moves in a continual stream toward their bases. Cholodny and Went postulated that auxin moves also from the upper to the lower side when a shoot is placed horizontally. As a result, the growth rates of the upper and lower halves would decrease and increase, respectively, and the shoot would curve upward. In 1927 Cholodny (13) generalized his hypothesis to cover the phototropic response, and in 1928 Went (28), again independently, arrived at the same explanation. The concept of hormonal coordination of plant tropisms has come to be called the Cholodny-Went Theory, in honor of both men. The development of the theory has been reviewed by Went and Thimann (29).

Several workers performed experiments which demonstrate that geotropic curvature of a shoot is the result of a decrease in growth of the upper half accompanied by an increase in growth of the lower half. Among these, the careful measurements of Navez and Robinson (20) on the Avena coleoptile seem particularly reliable.

Direct studies of auxin distribution following phototropic induction were first performed by Went in 1928 (28). He placed agar blocks basipetal to each half of a coleoptile tip which had been longitudinally split at the base. He illuminated the coleoptile unilaterally, perpendicular to the split, and, after a suitable length of time, measured the relative amounts of auxin in the two blocks by means of the Avena test. He found that the illuminated side released much less auxin than did the shaded side.

Shortly afterward, Dolk (15) performed similar experiments on both *Avena sativa* and *Zea mays* coleoptiles which had been induced by gravity. More auxin was obtained from halves which had been lowermost than from those which had been uppermost, while the total auxin yield of each organ was unaffected by stimulation. A number of workers repeated Dolk's experiment with other plant organs, and found similar distributions of auxin between the top and bottom halves (4, 14, 19, 20, 25). Other workers obtained similar distributions when auxin was obtained by extraction (5, 26).

Bünning et al (9), Gordon and Eib (18), Reisener (21), Reisener and Simon (22), and Ching and Fang (10), applying carbon 14-labeled indoleacetic acid to oat coleoptiles and to other plant parts, have recently been unable to demonstrate lateral movement of the labeled hormone during or following either geotropic or phototropic stimulation. Numerous other workers have expressed lack of confidence that lateral auxin transport occurs (1, 2, 3, 5, 11, 23), and some have proposed other mechanisms to explain the differential growth of the two sides (2, 7, 9). In view of the recent experiments of Briggs et al (8) suggesting that lateral transport of auxin may indeed mediate the phototropic response of coleoptiles of *Zea mays* to certain dosages of unilateral light, it seemed worth while to repeat these experiments, using gravity instead of light as the stimulus.

MATERIALS & METHODS

The methods for growing corn (*Zea mays* L. Burpee Snowcross) were almost identical with those described by Briggs et al (8). Auxin was collected from excised 7-mm corn coleoptile tips by setting 1.5 % agar blocks against the cut surfaces of the tips for 2.5 hours. Three similar tips were placed either vertically or horizontally against an agar block in order to determine whether or not the orientation of the tips with respect to gravity had any effect on the total amount of auxin diffusing from them. Next, six tips were partially bisected, leaving the apical 2 mm intact. The bases of each tip were separated by a glass cover slip, and agar blocks were set against the bases in such a way that auxin diffusing from bases on opposite sides of the glass could be collected separately. The assembly was placed so that the plane of bisection was horizontal. Halves from three completely bisected tips were laid horizontally with the convex side up, so that each would be comparable with the top half of a complete horizontal tip. Halves from three more bisected tips were laid horizontally with the convex side down, so that they would be comparable with the bottom halves of complete horizontal tips. In every experiment three intact tips were placed vertically on an agar block, and the

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amount of auxin obtained was thus used as a measure against which the percent recovery of auxin from the various experimental situations could be determined.

Following the auxin diffusion procedures, all agar blocks were assayed by means of the standard Avena curvature test (29). Control blocks containing 25 and 250 γ/liter indoleacetic acid were assayed with each test to determine whether or not the experimental values were within the proportionality range of the bioassay. The block containing 250 γ/liter was calculated to produce the maximum possible curvature obtainable in the time allotted for curvature development (110 min). Experience in this laboratory has indicated that as long as this curvature is in excess of 40 degrees, curvatures between about 5 and 30 degrees, obtained from other blocks assayed simultaneously, were directly proportional to auxin concentration.

Although manipulations were made rapidly in a room in which the humidity was 92 %, and the assemblies were placed immediately in petri dishes in which a saturated atmosphere was maintained, it was found desirable to keep the surface area of agar exposed to air the same in all cases. This was achieved by backing the upright blocks with glass. Water was added to the agar blocks to prevent drying. It tended to run off the vertical blocks, however, and to seep by capillary attraction between the coleoptile tips and the glass on which the tips rested. This problem was not serious for partially split tips, since they tended not to lie flat against the glass which separated their bases. In the case of the totally split coleoptiles, the drops of water placed on the blocks were small, and the tips were supported by a glass slide which did not touch the agar. The air gap between the agar and glass prevented water from being drawn out under the tips and carrying auxin away from the agar. If these precautions were not followed, recovery of auxin was reduced and the ratio of auxin between upper and lower halves was thus untrustworthy.

It might appear from this description that, since different amounts of water were applied to the receptor blocks of the various assemblies, the auxin yields from different assemblies should not be compared. However, the total amount of water added was regulated so that the same moisture content was maintained in each block, as far as could be determined visually. Furthermore, members of each pair of assemblies received identical treatment, so that the critical comparisons between upper and lower halves of partially split tips and between upper and lower halves of totally split tips are valid.

Results

The results are presented in tables I, II, and III. Each value represents the average degrees curvature of 12 Avena coleoptiles to which the blocks from a single auxin diffusion from three intact tips (or 6 half tips) were applied. The different numbered experiments were done on different days and the average of the standard errors for the values shown in these tables was ±2.0°; the standard error never exceeded ±3.3°.

Table I

Diffusible Auxin Obtained From Vertical & Horizontal Coleoptile Tips*

Expt.	Vertical tips	Horizontal tips
1	18.2	17.9
	18.3	18.9
	21.8	25.0
2	24.3	28.5
3	25.3	27.9
	29.8	28.2
	31.9	32.6
	35.1	35.8
4	39.7	36.1
Average:	27.2	27.9

* Degrees Avena curvature

Table I shows that equal amounts of auxin are obtained by diffusion from vertical and horizontal coleoptile tips. Although the range in the amount of auxin obtained from each set of three tips was considerable, and there were on some occasions statistically significant differences between amounts obtained from vertical and horizontal tips in a single experiment, these differences can be ascribed either to variability in the experimental material or to variability in technique from day to day. On different occasions either horizontal or vertical tips might yield the greater amount of diffusible auxin, and if the results of the several experiments are averaged, little difference between the yields is apparent. Thus Dolk's observations (15) are confirmed.

The distribution of diffusible auxin obtained from the upper and lower halves of partially split horizontal coleoptile tips is shown in table II. There

Table II

Distribution of Diffusible Auxin Obtained From Horizontal Partially Split Coleoptile Tips*

Expt.	Intact tips	Upper halves	Lower halves
5	29.2	20.6	33.2
6	28.0	17.4	39.8
	...	21.3	29.6
2	24.3	18.5	27.7
	28.5	...	...
4	39.7	16.8	36.9
	36.1	19.3	33.8
7	28.5	11.0	37.3
Average:	30.6	17.7	34.0

Per cent recovery from split tips: 85
Per cent of recovered auxin in upper halves: 34
* Degrees Avena curvature

is clearly far more auxin obtained from the lower than from the upper halves. Furthermore, in every case, the difference was clearly significant. It should be noted that only 85 % as much auxin was obtained, on the average, from horizontal split tips as from vertical intact ones. It might well be argued that the 15 % loss was entirely from the upper sides. Even if this had been the case, however, inspection of the table reveals that the difference between yields from upper and lower halves is still significant. Indeed, it seems far more likely that the loss is an artifact reducing the apparent amount of auxin obtained from the lower halves, since many of the values shown for the lower halves are somewhat beyond the proportionality range of the bioassay.

Finally, the distribution of diffusible auxin obtained from totally split horizontal coleoptiles is shown in table III. The averages shown indicate that approximately equal auxin quantities are obtained from upper and lower halves. Although the differences between the halves are sometimes significant, they can again be ascribed to technical causes since they do not consistently appear, and may be in either direction. In no case are they of the magnitude of those found between upper and lower halves of partially split tips.

TABLE III

DISTRIBUTION OF DIFFUSIBLE AUXIN OBTAINED FROM HORIZONTAL TOTALLY SPLIT COLEOPTILE TIPS*

EXPT.	INTACT TIPS	UPPER HALVES	LOWER HALVES
2	24.3	15.6	22.6
4	39.7	29.0	33.9
	36.1	18.8	26.8
	...	28.5	23.8
8	28.5	18.8	22.3
	...	23.8	22.8
Average:	32.2	22.4	25.4

Per cent recovery from split tips: 74
Per cent of recovered auxin in upper halves: 47
* Degrees Avena curvature

The lateral gradient of diffusible auxin in geotropically stimulated coleoptiles (table II) and the equality of total diffusible auxin production in stimulated and nonstimulated coleoptiles (table III) suggest that auxin is not being produced or destroyed as a consequence of a geotropic stimulation, but rather that auxin or an auxin precursor is moving from the upper to the lower sides. This suggestion is supported by the demonstration that complete separation of lower and upper sides virtually eliminated the appearance of an auxin gradient across the coleoptile. These results are closely in accord with those of Briggs et al (8) for phototropically stimulated coleoptiles.

DISCUSSION

The evidence above might be used to support the alternative hypothesis that an inhibitor moves from the lower to the upper side of a stimulated coleoptile. Unless a special undiscovered polar transport system is postulated for such an inhibitor, it must be assumed that this agent operates by releasing auxin into the auxin transport system in the lower side and by removing an equal amount of auxin from the transport system on the upper side. Such a hypothesis seems unlikely, since it demands the existence in the corn coleoptile of an inhibitor with curious properties unlike any which have been reported in the literature, and since it is no easier to understand why an inhibitor should be laterally translocated than why auxin should be laterally translocated. Similar reasoning argues against the participation of an auxin synergist.

The further possibility of lateral translocation of a substance participating in auxin synthesis in some capacity other than that of an immediate precursor is made unlikely by Dolk's observation: when auxin, collected in agar from corn coleoptile tips, is permitted to diffuse from the agar into horizontal decapitated oat coleoptile sections, more auxin may be obtained from agar receptor blocks which have been applied to the lower basal cut surface of the oat coleoptile sections than from blocks applied to the upper basal surface (15).

If the results of these experiments are valid, the failure of several workers to obtain evidence for lateral transport of exogenous radioactive indoleacetic acid must be explained. In some cases (10, 21, 22), organs with the tips intact were submerged in a solution of radioactive auxin. Following geotropic stimulation, the distribution of the auxin removed from solution by the organ was studied by measuring the radioactivity of the tissue. It was never proven in these experiments that all or even a substantial fraction of the labeled auxin entered the transport system. Yet it was shown in Dolk's experiments and in all experiments modeled after these that, in coleoptiles, it is auxin moving in the transport system which is correlated with tropic behavior. If the auxin in the transport system is partially endogenous and partially exogenous, and if a relatively large amount of exogenous auxin is present in the tissues without participating in transport, any lateral redistribution of moving auxin would be extremely difficult to detect by measuring tissue radioactivity, considering that the absolute quantities are exceedingly small.

On the other hand, some workers (9) decapitated coleoptiles and supplied labeled auxin at the cut surface. Following phototropic stimulation auxin was again assayed by measuring tissue radioactivity. It has been demonstrated that labeled auxin supplied in this manner enters the transport system (17). However, it has also been demonstrated that, in corn,

phototropic lateral redistribution probably occurs entirely within less than 0.4 mm of the coleoptile tip and possibly within less than 0.2 mm (W. R. Briggs, unpublished). An oat coleoptile is capable of perceiving and reacting to gravity along its entire length. However, the greater part of the lateral transport occurs in the top 3 mm; more basal regions can effect only a slight redistribution (15). In view of all these considerations, studies of redistribution of labeled auxin in decapitated coleoptiles are of doubtful value, unless they measure only auxin in the transport system. The best way to do this is to measure labeled indoleacetic acid diffusing from coleoptile tips into agar blocks. Indeed, experiments by Gillespie and Thimann (16) clearly show that radioactive indoleacetic acid applied in agar blocks to the apical ends of Avena coleoptile sections is laterally redistributed by gravity in horizontal sections, but that such redistribution is restricted to that portion of the applied auxin which has actually entered the transport system (in their experiments, less than 20 % of the total entering the tissue). It can be demonstrated clearly only by using auxin diffusion techniques.

Brauner and Appel (6) have recently published experiments which are particularly pertinent to the present discussion. They found that if the apices of detached Avena coleoptiles were split to a depth of 2 mm, and a small piece of mica inserted into the split, subsequent geotropic response was far lower if the coleoptiles were induced with the split horizontal than with the split vertical, suggesting blockage of some transport pathway by the mica in the horizontal case. Although they did indeed obtain some curvature with the mica horizontal, this is not surprising since Dolk (15) has shown that a certain amount of geotropic induction is possible below the apex itself.

These authors also demonstrated that if half the apex of detached coleoptiles is excised by a vertical and a horizontal cut, and agar blocks appressed against the remaining vertical cut surfaces, significant amounts of auxin could be collected in such blocks (as measured by the Avena curvature test) if the coleoptiles were geotropically stimulated with the blocks on the lower side, but not if they remained vertical. Their experiments thus suggest that gravity induces the transverse migration of auxin itself, rather than a precursor, since the activity of the transversely migrating substance is detected immediately as auxin in the Avena curvature test. A lag in such a test would have suggested the presence of a precursor.

The fact that geotropic response is mediated by lateral transport in the corn coleoptile does not mean that lateral transport is the only mechanism by which plants respond to gravity. For example, the amount of auxin which can be obtained from grass nodes (24) and from the meristematic growth rings of sugar cane nodes (26) increases when these plants are placed horizontally. There is no evidence, however, that auxin production or release is unilateral; lateral transport may concentrate the newly appeared auxin in the lower half.

Summary

The distribution of auxin during geotropic stimulation of corn coleoptiles has been determined by means of the Avena test. It has been verified that equal amounts of auxin diffuse out from vertical and from horizontal coleoptile tips, and that the lower halves of partially split coleoptile tips release a larger amount of auxin than do the upper halves. When coleoptile tips are completely bisected prior to stimulation, however, the same quantity of auxin is obtained from each half. The simplest interpretation of these results is that auxin, or possibly an auxin precursor, is translocated from the upper to the lower side of a stimulated coleoptile.

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INVOLVEMENT OF ADENYLATE KINASE IN PHOSPHORYLATION OF RIBOSE-5-PHOSPHATE BY ADENOSINEDIPHOSPHATE IN MITOCHONDRIA-FREE PREPARATIONS FROM ROUGH LEMON LEAVES[1]

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In some of our previous studies it was noted that ADP[2] was effective as the ATP source in crude homogenates for the carboxylation enzyme system (4) when R5P was used as substrate. The overall reaction is as follows:

$$R5P + ATP + HCO_3^- \xrightarrow{Mg^{++}} 2\ PGA + ADP$$

This reaction is itself a 3-enzyme sequence (6, 8). Studies were undertaken to elucidate the nature of the ADP replacement of ATP. Specifically, phosphorylation of ADP by P_i, by labile phosphates such as creatinophosphate and acetylphosphate, and by an adenylate kinase were investigated as possible means of formation of ATP from ADP in the preparations.

MATERIALS & METHODS

The K salts of R5P and G6P were prepared from the Ba salts by precipitating the Ba with an equivalent amount of K_2SO_4, and centrifuging at 1,000 × *g* at 0° C to remove the precipitate. Stock solutions of 0.03 M were prepared in water which had been demineralized in a Barnstead Bantam demineralizer. The K salt of acetylphosphate was prepared similarly from the di-Ag salt using an equivalent amount of KCl to precipitate the Ag.

In all the experiments rough lemon leaves (*Citrus jambhiri* Lush.), washed in distilled water, dried on paper towels and with the midrib removed, were used in the ratio of 2 g fresh weight of leaves per 5 ml tris-sucrose buffer (0.2 M tris plus 0.5 M sucrose, pH 8.0) for the homogenates. After grinding the leaves with a Virtis homogenizer at 75% maximum speed for 1 minute, the homogenate was strained through a double layer of cheesecloth, centrifuged at 1,000 × *g* for 10 minutes at 0° C to remove unbroken cells, cell debris, and large particles. A mitochondria-free supernatant was obtained by centrifuging the crude supernatant at 30,000 × *g* at 0° C for 20 minutes.

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[2] Abbreviations used in this paper: AMP for adenosinemonophosphate; ADP for adenosinediphosphate; ATP for adenosinetriphosphate; PGA for 3-phosphoglycerate; R5P for ribose-5-phosphate; G6P for glucose-6-phosphate; tris for tris(hydroxymethyl)aminomethane; P_i for inorganic phosphate; EDTA for ethylenediaminetetraacetic acid.

The reaction mixtures contained 20 μmoles $MgSO_4$ and 2 μmoles EDTA per ml. When used, 9 μmoles R5P, 6 μmoles G6P, acetylphosphate and creatinophosphate, and 100 μmoles $HC^{14}O_3^-$ (specific activity 13,000 counts/min/μmole) were added per ml reaction mixture. On adding 0.4 ml of the homogenate they were incubated at 37° C in a constant temperature metabolic shaker for 30 minutes, after which period the enzymes were inactivated by adding 0.3 ml 50 % trichloracetic acid giving a final volume of 3.0 ml. The acid also removed unreacted HCO_3^- as CO_2 which was eliminated by passing a stream of air over the reaction mixture. In the carboxylation experiments the reaction mixtures were centrifuged at 1,000 × *g* for 2 minutes and a 0.4 ml aliquot of the supernatant was dried under forced air at room temperature on a pyrex glass planchet. The samples were then counted with a Q-gas flow counter which was attached to an automatic sample changer and printer. Self absorption was corrected for, according to the weight of the dried sample. In experiments to determine adenylate-phosphate reaction products, the reaction mixtures were centrifuged at 1,000 × *g* for 2 minutes and the supernatant transferred to a centrifuge tube. Ten milliliters of ether were added, the mixture shaken, and the two phases allowed to separate. The ether layer was removed by aspiration and the ether extraction repeated. One drop of 2 % NH_4OH was added to the aqueous solution before adding a 2 × 2 cm^2 of SA2 amberlite ion exchange paper to remove excess Mg^{++} ions. The solutions were shaken for 5 minutes and then suitable amounts (50–300 μl) depending on the concentration of adenylate-phosphate added per reaction mixture, were applied in several successive applications on Whatman 3 MM chromatography paper which had been washed successively with 0.02 M EDTA, 2 N acetic acid, 0.1 N HCl and demineralized water.

Descending chromatographs using *n*-propylacetate, 90 % formic acid and water in a 11 : 5 : 3 ratio by volume, were run for 6 hours, after which they were dried at room temperature. Nucleotide spots were located under ultraviolet light (Mineralite, Model SL 2537) and identified by comparison with known standards. The nucleotides were eluted by placing the strip of paper containing the spot on a larger strip of tin foil, saturating the paper strip with demineralized H_2O and rolling the two strips lengthwise so that the paper strip was completely contained in the tin foil strip. These rolls were centrifuged at 1,000 × *g* for 5 minutes and the solutions obtained made up to 3 ml with demineralized water. Aliquots of these solutions were placed in silica cuvettes and the OD measured at 260 mμ, using a slit width of 0.2 mm, in a Beckman Model DU spectrophotometer. Standard curves for adenosine, AMP, ADP, and ATP were obtained for determination of concentration of the various nucleotides. Inorganic phosphate was determined by the method of Bernhart and Wreath (1).

TABLE I

RELATIVE AMOUNT OF $C^{14}O_2$ FIXATION AT VARYING ATP & ADP LEVELS

ADENYLATE-PHOSPHATE LEVEL	μmole $C^{14}O_2$ FIXED PER ml REACTION MIXTURE		$C^{14}O_2$ FIXATION ADP/ATP	% EFFICIENCY	
μmole/ml	ATP	ADP	%	ATP	ADP
½	1.01	0.45	45	202	90
1	1.90	0.86	45	190	86
2	3.67	1.68	46	184	84
4	4.93	2.17	44	123	54

All studies were repeated a sufficient number of times to assure the points that were established.

RESULTS & DISCUSSION

Replacement of ATP by ADP at four levels of each in the carboxylation reaction was shown to be 45 % (table I). As seen in table II, the addition of an equimolar amount of P_i to ADP decreased the fixation of CO_2 indicating no phosphorylation of ADP by P_i in the mitochondria-free supernatant. Similarly, there was no increase in fixation of CO_2 on the addition of 6 μmole of G6P, acetylphosphate or creatinophosphate per ml reaction mixture, respectively, indicating that labile phosphates were not responsible for the replacement of ATP by phosphorylation of ADP. These results were borne out by the data in table II where the amounts of ATP formed were measured in reactions excluding R5P and HCO_3^-. Neither P_i, G6P, acetylphosphate or creatinophosphate increased the amount of ATP formed.

ATP could be formed by an adenylate kinase (2, 7) in the following reaction:

$$2\ ADP \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} ATP + AMP$$

This was confirmed by the results in table III where ATP and AMP were formed from ADP. Theoretically, the amounts of ATP and AMP formed

TABLE II

$C^{14}O_2$ FIXATION & PHOSPHORYLATION OF ADP BY P_i, G6P, ACETYLPHOSPHATE & CREATINOPHOSPHATE

ADP	OTHER	$C^{14}O_2$ FIXATION LESS CONTROL	ATP FOUND AFTER INCUBATION WITHOUT R5P & HCO_3^-
μmole/ml reaction mixture		μmole/ml reaction mixture	μmole/ml reaction mixture
4	...	1.94	0.59
4	4 Pi	1.63	0.50
4	6 G6P	1.68	0.45
4	6 AcP	0.62	0.17
4	6 CrP	1.72	0.56

TABLE III

$C^{14}O_2$ FIXATION & REACTION PRODUCTS FORMED FROM 2 μmoles ADENYLATE PHOSPHATE WITH & WITHOUT R5P & HCO_3^-

REACTION MIXTURE	$C^{14}O_2$ FIXATION LESS CONTROL	μmole/ml FOUND AFTER INCUBATION				
		ADENOSINE	AMP	ADP	ATP	P_i
	μmole/ml reaction mixture					
ATP + R5P + HCO_3^-	3.67	0.48	0.97	0.36	0.17	1.54
ATP	...	0.92	0.21	0.25	0.74	2.84
ADP + R5P + HCO_3^-	1.68	0.61	1.08	0.21	0.16	0.98
ADP	...	0.93	0.68	0.23	0.20	1.56
AMP + R5P + HCO_3^-	0.06	0.63	1.33	0.00	0.00	0.65
AMP	...	1.23	0.68	0.04	0.04	0.84
Control	...	0.00	0.00	0.00	0.00	0.00

should be equal. However, it is evident from the reaction where ATP alone was added that a phosphatase was active which gave ADP from ATP. This could account for the low value obtained for ATP.

In reactions where a relative excess of R5P and HCO_3^- was added, there would be a competition for ATP between the phosphatase and phosphopentokinase. That this latter enzymatic process predominated was evident from the amount of P_i formed in the reactions where R5P and HCO_3^- were added as compared to those where they were omitted.

The adenylate kinase reaction can explain the efficiencies of ATP and ADP in the reactions resulting in $C^{14}O_2$ fixation in table I. ADP formed from the ATP phosphorylation of R5P was cycled to ATP and AMP. As ATP was removed by phosphorylation of R5P the ATP or ADP would eventually end all as AMP if the reactions went to completion. The 200 % efficiency for ½ μmole ATP in $C^{14}O_2$ fixation in table I and the 90 % for ½ μmole ADP indicated this. This is consistent with the published equilibrium constant for adenylate kinase (3).

The two reactions could then be coupled as follows:

$$2\ ADP \rightleftharpoons ATP + AMP$$

$$R5P + ATP + HCO_3^- \xrightarrow{Mg^{++}} 2\ PGA + ADP$$

with the overall reaction:

$$R5P + ADP + HCO_3^- \xrightarrow{Mg^{++}} 2\ PGA + AMP$$

From table III it can be seen that corresponding results were obtained both by CO_2 fixation and by measurement of adenylate reaction products. Production of P_i occurred as a result of an AMP phosphatase. However this did not account for all the P_i formed. P_i could also be formed by the action of an ATPase or possibly by the removal of a phosphate group from a reaction product.

There was no evidence that AMP and ADP reacted to yield ATP plus adenosine.

SUMMARY

Studies were made of CO_2 fixation in mitochondria-free preparations of rough lemon leaves using R5P as a substrate with ATP generated within the system from ADP. P_i, G6P, acetylphosphate and creatinophosphate were inactive phosphorylating agents. Both CO_2 fixation studies and measurement of adenylate phosphate end products gave essentially the same results and indicated an adenylate kinase to be the mechanism whereby ATP was formed from ADP. The cycling nature of this enzyme resulted in a 200 % efficiency for $C^{14}O_2$ fixation with ATP and 90 % with ADP at low levels of ATP and ADP. The results also indicated a number of phosphatases to be present in the system.

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AN ESTIMATE OF A MINIMUM QUANTUM YIELD OF PHOTOSYNTHESIS BASED ON ECOLOGIC DATA[1]

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Recent investigations of forest productivity by Mar: Möller (10), Mar: Möller et al (11), Polster (18), and Ovington and Heitkamp (15) show higher efficiencies in relation to yearly solar energy supply than have been reported in the same geographic area for herbaceous communities and crop plants. Since the results of these studies are in, or can be converted into, energy units, they can be used in calculating a minimum value of the quantum yield of photosynthesis. An estimate will be made of the quantum yield for the most efficient forest thus reported, the *Picea omorika* Mast. plantation of Ovington and Heitkamp.

PRODUCTIVITY

The caloric content of material sampled from the above ground portion of the *Picea omorika* stand at 21 years was determined, using an oxygen bomb calorimeter, as $15{,}560 \times 10^8$ g cal./ha. (15). Above ground accumulation was, therefore, 740.9×10^8 g cal./ha./year. Ovington and Heitkamp estimated root accumulation as around 28 % of the above ground accumulation, or 205.5×10^8 g cal./ha./year. Total yearly accumulation was 946.4×10^8 g cal./ha. Ovington (14) in a study of a series of *Pinus sylvestris* L. plantations and Ovington and Madgwick (16) in natural stands of *Betula alba* L. found that at 20 years, the annual production of organic matter was approximately double the amount accumulated. Non-accumulated production was used in respiration, consumption by animals, and in loss of organic matter removed as crop or decomposed on the forest floor. Ovington and Heitkamp estimated that if the same ratio of production to accumulation applied to the *Picea omorika* plantation, the total productivity at 21 years was $1{,}893 \times 10^8$ g cal./ha./year.

SOLAR RADIATION

The mean direct solar radiation on a surface normal to the sun at Kew Observatory (51° 28′N, 00° 19′W) from 1933 to 1958 (data not obtained for 1947, 1948, 1956) was $57{,}000 \times 10^8$ g cal./ha./year (1, 21). The Kew Observatory is in the near vicinity of the meteorological station mentioned by Ovington and Heitkamp (Lympne 51° N 0°) as being close to the *Picea omorika* plantation.

The amount of energy within the wave lengths of the action spectrum of photosynthesis (ca. 390–760 mμ) is usually stated as around 50 % of total radiation. The accuracy of this value was checked for the *Picea omorika* stand area as follows: The distribution of total solar radiation on a monthly basis for an area 52° N 10° W was procured from Kimball (6). The mean zenith distance of the sun from 9h to 15h was determined on a monthly basis from data in table 170 of List (9) for 50° N latitude. The period 9 to 15h was chosen since the vast majority of solar radiation is received within this period, and no data were available on hourly radiation flux to allow hourly corrections. The mean monthly optical air mass was calculated for the given zenith distances from table 137 of List. The energy content of radiation within 390 to 760 mμ was then calculated on a monthly basis as a percentage of total radiation from values in table 148 of List. The mean yearly receipt of energy within 390 to 760 mμ was found to be 49.7 % of the energy content of total radiation at earth surface (for 50° N). The light energy within the action spectrum of photosynthesis for the *Picea omorika* stand was, therefore, $28{,}329 \times 10^8$ g cal./ha./year.

A deliberately wide range for the action spectrum of photosynthesis was chosen to insure a subsequent minimum estimate of quantum yield. Had a narrower range been chosen, for example from 400 to 700 mμ, the amount of energy as a percentage of total radiation would have been 44.3 (for 50° N).

Of visible radiation striking the forest, less than 5 % is reflected back to the sky. Measurements of visible albedo at a given instant during the day include values of 2.5 % for a *Pinus resinosa* Ait.—*Pinus strobus* L. forest (Bray, unpublished data); 2.9 % for an Abies forest (8); 3.0 % for a *Larix laricina* (Du Roi) K. Koch forest (Bray, unpublished data); 3.4 and 3.6 % for a conifer forest (7). Since albedo varies with solar zenith distance, single albedo measurements are only a broad estimate of the mean albedo for the daily period. No data are available for mean daily albedo of a conifer forest; a *Quercus ellipsoidalis* E. J. Hill forest had a mean daily albedo of 2.8 % (Bray, unpublished data). If a minimum estimate of 2.5 % visible albedo is made for the

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Picea omorika plantation, the visible light penetrating the stand equals 27,621 × 10^8 g cal./ha./year.

A minimum estimate of light penetrating the *Picea omorika* stand which reaches the forest floor and is absorbed there can be made from the notation by Ovington and Heitkamp, "The almost complete absence of a ground flora in the spruce stand is indicative of the efficiency of the spruce trees in intercepting most of the incident light energy". Mitscherlich (12) reported for Picea plantings that light penetration for young stands up to 30 years was 7 to 10 % of outside intensity. In these stands the forest floor was bare of plants. Nägeli (13) reported that by the end of September the light penetration on the floor of a pure Abies forest was 4 % of outside light indicating an approximate crown absorption of 96 %. Bray (unpublished data) found a 96 % crown absorption (which incorporated reflectance from the forest floor) for a *Quercus ellipsoidalis* forest. Trapp (22) measured a crown absorption of 95 % in a *Fagus sylvatica* L. forest. Utilizing the maximum crown absorption of 96 %, the total light absorbed by the vegetation is 26,516 × 10^8 g cal./ha./year. This absorption occurs in both leaf and stem tissue, the vast majority occurring in the leaves of the crown. Regardless of the amount of light absorption by stems, this absorption cannot be subtracted from the amount available to photosynthetic tissue since bark and woody tissue of tree stems contain chlorophyll (Gunderson 5, Pearson & Lawrence 17, Bray 2). Bray found a total of 0.24 g of chlorophyll a and b for the stem tissue of a *Pinus banksiana* Lamb. tree which was 15.3 m in height. The stem chlorophyll was 2.8 % of the total chlorophyll in the tree.

Of the visible radiation absorbed by the photosynthetic tissue, a portion is absorbed by non-pigmented cellular parts. Seybold (19) estimated that this portion is 10 % or less of the total light absorbed by leaves. Seybold and Weissweiler (20) measured light absorption in chlorophyll-free leaves of *Pelargonium zonale* L'Herit. and found from 18 to 20 % absorption from 600 to 700 mμ. Assuming a value of 10 % for a minimum non-pigmented absorption, the total visible radiation available to the pigments of the photosynthetic tissue is approximately 23,864 × 10^8 g cal./ha./year.

The energy budget described above is summarized in table I.

Efficiency

The total energy transformed in photosynthesis was estimated for the *Picea omorika* stand as 1,893 × 10^8 g cal./ha./year. The total visible radiation (390–760 mμ) reaching the pigments of the photosynthetic tissue was 23,864 × 10^8 g cal./ha./year. The efficiency of utilization of absorbed light is 7.9 %.

Quantum Yield

The quantum yield of photosynthesis (ϕ) as derived from equation 4a of Gaastra (4) is:

$$\phi = \frac{\epsilon \text{ absorbed} \times (2.85/\lambda)}{112{,}000}$$

where λ = wave length (cm) and ϵ absorbed = the efficiency of true photosynthesis for absorbed light. Values of ϕ were calculated of 0.047 and 0.030 for wave lengths of 430 and 660 mμ which approximate the absorption peaks of chlorophyll a. The mean of these values, ϕ = 0.038, is a minimum approximation for non-monochromatic light over the range 390 to 760 mμ. A maximum approximation can be made by choosing those energy supply values which result in the highest quantum yield. This approximation is 0.053 for the energy supply values listed above over the range of 400 to 700 mμ.

The energy requirement of photosynthesis ($1/\phi$) for the plantation ranges from 19 to 26. This is an estimate for the entire year for both leaf and stem tissue and, as could be expected, is higher than the estimates made since 1950 reported in Daniels (3) which are below 15. Daniels calculated a value of ca. 15 for tomato plants grown under optimum green-

Table I

Estimated Energy Kinetics of Picea Omorika Plantation of Ovington & Heitkamp

	Gram calories per hectare per year
Accumulated above-ground vegetation	740.9 × 10^8
Accumulated below-ground vegetation	205.5 × 10^8
Consumed vegetation	946.4 × 10^8
Produced vegetation	1,892.8 × 10^8
Incoming total solar radiation	57,000 × 10^8
Incoming visible radiation (390–760 mμ)	28,329 × 10^8
Visible radiation penetrating plantation	27,621 × 10^8
Visible radiation penetrating photosynthetic tissue	26,516 × 10^8
Visible radiation available to photosynthetic pigments	23,864 × 10^8
Efficiency of photosynthesis in relation to visible radiation available to photosynthetic pigments	7.9 %

house conditions from data in Went (23). For field crops, the lowest reported $1/\phi$ value for the most favorable part of the growing season is 10 (Gaastra, 4) for sugar beets. *Picea omorika* leaves at the most favorable time of day and growing season must be considerably below the 19 to 26 range for the plantation over the year.

Summary

An estimate is made of the quantum yield of photosynthesis from information on organic matter production of a forest and its estimated solar radiation energy budget. Data based on caloric content of a *Picea omorika* plantation show above ground accumulation of 740.9×10^8 g cal./ha./year, total accumulation of 946.4×10^8 g cal./ha./year and total production of 1,893 g cal./ha./year. Direct solar radiation is $57{,}000 \times 10^8$ g cal./ha./year. Analysis of radiation measurements in conifer stands suggest a maximum visible radiation of $23{,}864 \times 10^8$ g cal./ha./year is available to the photosynthetic pigments. Efficiency in relation to visible radiation is 7.9 %. Minimum quantum yield estimate for the plant community over the year is 0.038; maximum estimate is 0.053. The yield for leaf tissue at the most favorable time of day and season is presumably much higher.

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CARBON DIOXIDE FIXATION BY AUXIN TREATED TISSUES [1, 2]

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The relationship between CO_2 fixation and growth is a matter of considerable interest. Molliard (6) was probably the first to report that CO_2 is beneficial for the elongation of pea stems and lupin hypocotyls in darkness as well as in light. Very little attention was paid to this observation until Yamaki (13) demonstrated that a 100 to 200 % increase in elongation of Avena coleoptiles was effected when as little as 0.5 % CO_2 was present in the experimental atmosphere. Similar promotions of growth in coleoptiles and internodes of Avena have also been obtained by Nitsch and Nitsch (8) in certain experiments. More recently Mer (4, 5) has shown that in intact seedlings a concentration of 5 % CO_2 promoted mesocotyl growth but inhibited the extension growth of coleoptiles. This inhibition is overcome by nitrates supplied to the seedlings.

An investigation was therefore undertaken to study the products of $C^{14}O_2$ fixation in relation to auxin-induced growth and inhibition in coleoptile sections as also in intact plants. This paper is concerned with a study of the changes occurring in the detached coleoptile.

During the progress of this investigation a brief account of Yamaki's recent work was made available (14). His conditions were different from those employed by us but there is a large measure of agreement in the conclusions drawn, namely, that IAA stimulates CO_2 fixation and has a marked influence on the metabolism of the fixation products.

MATERIALS & METHODS

The variety of oat used was N.P. 1 obtained from the Indian Agricultural Research Institute, New Delhi. Husked seeds were germinated in rows on moist filter paper in a chamber with occasional red light. The temperature of the chamber was maintained at 24° C. After 72 to 96 hours the coleoptiles were decapitated by about two millimeters and floated on distilled water in darkness for 3 hours to free them from endogenous auxins. Six mm sections were transferred to McIlvaine's phosphate buffer solution of pH 7.0, containing $NaHC^{14}O_3$, and specified amounts of addenda. The control received an equivalent amount of distilled water so that the final volumes were the same in all containers. Incubation was in darkness for durations specified. For root studies 10 mm sections were used.

The reaction was usually stopped by adding hot 80 % ethanol. The tissues were twice extracted with hot 80 % ethanol and filtered. The filtrate was evaporated to dryness, dissolved in a small volume of water, and chromatographed two-dimensionally with phenol-water as the first and butanol-acetic acid-water (4 : 1 : 1) as the second solvent. The radioactive spots were located by exposure to Ilford or Kodak X-ray films. Counts were taken directly on the paper with an end-window Geiger-Müller counter. The compounds were identified by migration characteristics in a variety of solvents and co-chromatography wherever possible. Aliquots of the extracts were also counted in stainless steel planchets after several treatments with acetic acid and evaporation to dryness to get rid of any unreacted $NaHC^{14}O_3$. The cell-wall constituents were fractionated following essentially the method of Boroughs and Bonner (1).

Homogenates of the coleoptiles were prepared after the procedure of Price and Thimann (9). DPNH[3] and TPNH were extracted according to the method outlined by Colowick and Kaplan (2). DPNH was estimated by following the changes in optical density at 340 mμ on a Beckman model DU spectrophotometer directly or by coupling to enzymatic assays. The enzymes used were: A, yeast alcohol dehydrogenase with acetaldehyde as the substrate in pyrophosphate buffer of pH 8.5 and B, rabbit muscle lactic dehydrogenase (contaminated with PEP Kinase) with pyruvate as the substrate in phosphate buffer of pH 7.5. The enzymes were very kindly donated by Dr. D. P. Burma of this Institute. $NaHC^{14}O_3$, *l*-aspartic acid-G-C^{14} and glutamic acid-1-C^{14} were obtained from the Radiochemical Centre, Amersham, England.

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[2] The investigations were carried out during the years 1956 to 1959, aided in part by a grant from the Ministry of Education, Government of India.

[3] The following abbreviations have been used: IAA, indole-3-acetic acid; NAA, naphthalene acetic acid; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; CoA, coenzyme A; AMP, adenosine monophosphate; ATP, adenosine triphosphate; PEP, phosphoenolpyruvate.

Results & Discussion

Preliminary experiments indicated that $C^{14}O_2$ fixation by Avena coleoptiles in the absence of IAA was very small. The use of $NaHC^{14}O_3$ ensured a greater uptake of C^{14} and in all subsequent experiments $NaHC^{14}O_3$ of a specific activity of 103 μc/mg was used. $C^{14}O_2$ fixation was indicated by a decrease in the total carbon content of the surrounding solution. That Avena coleoptiles fix CO_2 in darkness has also been observed by earlier workers (10, 13).

The results of an experiment in which the coleoptiles were incubated with $NaHC^{14}O_3$ (40 μc/ml of experimental solution) in the presence of different concentrations of IAA for 15 hours in darkness are presented graphically in figure 1. It is clear that auxin stimulated the incorporation of C^{14} into the alcohol-soluble fraction of the coleoptiles but the quantity in the insoluble fraction was practically unaffected. It will be observed that 10^{-5} M is the optimum concentration. At higher concentrations the incorporation of C^{14} declined. In a parallel experiment in which non-radioactive $NaHCO_3$ of a similar concentration was used the auxin-induced growth rate was found to attain a maximum also at 10^{-5} M, but the increased growth rate was retained even at 10^{-4} M, though CO_2 fixation decreased at this concentration. Yamaki (13) observed that soon after the addition of IAA the volume of CO_2 consumed increased considerably; when, however, acceleration of elongation took place, there was a decrease in CO_2 fixation.

Radioautograms of the alcohol soluble fraction revealed the presence of C^{14} in a variety of compounds (fig 2). Among the organic acids citric-isocitric, malic, fumaric, and succinic acid were detected. Considerable radioactivity was detected in another compound which has been identified as the ethyl ester of malic acid, probably an artifact formed during extraction with hot ethanol. Among the amino acids and amides an accumulation of C^{14} was also observed in aspartic acid, glutamic acid, and asparagine.

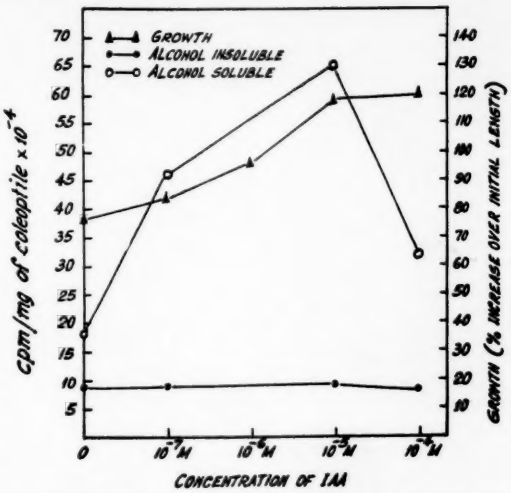

FIG. 1. The effect of IAA on the incorporation of C^{14} (from $NaHC^{14}O_3$) and on growth of Avena coleoptile sections.

When IAA was added to experimental solutions on which coleoptiles were floated no new compounds were detected but the distribution of C^{14} into the compounds was markedly affected (table I). Thus IAA stimulated the incorporation of C^{14} into citrate-isocitrate; but this fraction constituted a smaller portion of the total C^{14}-activity with IAA than without. C^{14}-activity in asparagine also increased and with such low concentrations as 10^{-7} M it was almost doubled; higher concentrations had a retarding effect on this stimulation. One of the most remarkable changes was the sharp fall of radioactivity in aspartic acid; it dropped from 27 % to less than 1 %. Most marked change, however, was in the large accumulation of C^{14} in malic acid. In untreated coleoptiles it constituted only 7 % of the total radioactivity. On addi-

TABLE I

EFFECT OF IAA ON PRODUCTS OF $C^{14}O_2$ FIXATION IN AVENA COLEOPTILES (TIME 15 hr cpm $\times 10^{-3}$)

COMPOUNDS	No IAA		10^{-7} M IAA		10^{-5} M IAA		10^{-4} M IAA	
	cpm/mg	% TOTAL	cpm/mg	% TOTAL	cpm/mg	% TOTAL	cpm/mg	% TOTAL
Citrate-isocitrate*	66.6	40.5	156.9	37.5	192.4	32.1	77.1	26.4
Phosphoenol pyruvic acid	6.1	3.7	2.4	0.6	3.1	0.5	8.1	2.8
Malic acid	11.6	7.1	180.6	43.2	300.1	50.1	154.5	52.9
Fumaric acid	5.3	3.3	17.2	4.1	4.0	0.7	4.8	1.6
Asparagine	8.2	5.0	46.4	11.1	36.6	6.1	22.0	7.6
Aspartic acid	45.1	27.5	3.2	0.8	+	+	1.9	0.6
Glutamic acid	1.6	1.0	3.0	0.7	3.1	0 5	4.1	1.4
Alanine	1.0	0.6	+	+	+	+	+	+

* Contaminated with the ethyl ester of malic acid.
+ Traces.

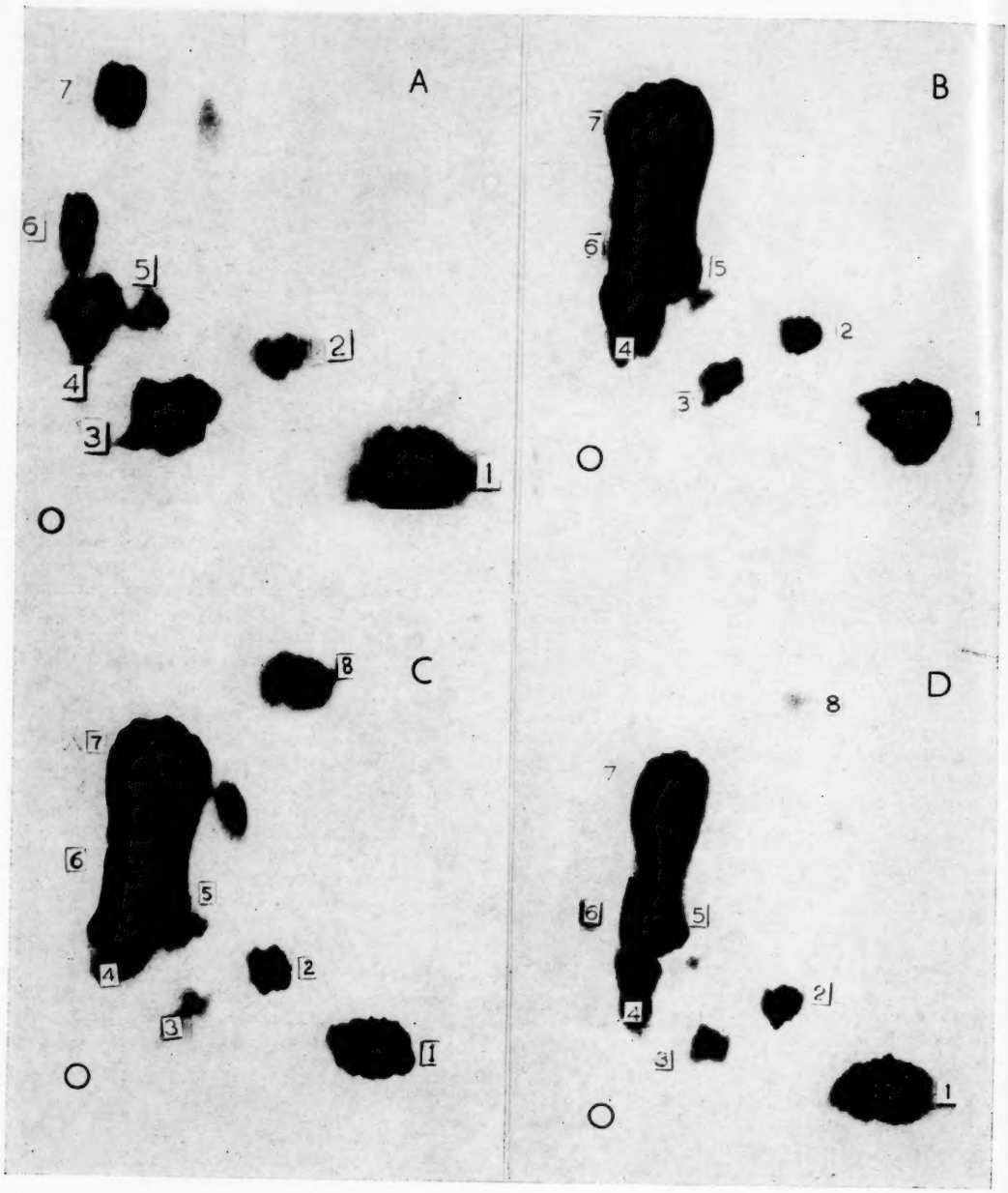

FIG. 2. Radiochromatograms of the products of $C^{14}O_2$ fixation (supplied as $NaHC^{14}O_3$) by Avena coleoptiles as affected by different concentrations of IAA during a 15 hr incubation period. A, Control; B, 10^{-7} M IAA; C, 10^{-5} M IAA; and D, 10^{-4} M IAA. Compounds: 1, asparagine; 2, glutamic acid; 3, aspartic acid; 4, citric-isocitric acid; 5, ethyl ester of malic acid; 6, phosphoenol pyruvic acid; 7, malic acid; 8, succinic acid. The chromatograms are developed from the origin (circle at lower left) with phenol-water to the right and butanol-acetic acid-water upwards.

TABLE II

EFFECT OF IAA ON INCORPORATION OF C^{14} INTO 80 % ETHANOL SOLUBLE & INSOLUBLE FRACTION OF AVENA COLEOPTILES ALLOWED TO METABOLIZE $NaHC^{14}O_3$ FOR 20 MINUTES (cpm/mg OF COLEOPTILE)

FRACTION	CONTROL	IAA TREATED
80 % ethanol soluble	3,532	9,271
80 % ethanol insoluble	825	808

tion of a little auxin (10^{-5} M) and above, half of the total radioactivity in the 80 % ethanol soluble fraction accumulated in malate.

In subsequent experiments, the period of experimental duration was reduced to shorter periods to study the effect of IAA on CO_2 fixation during the early stages (table II). CO_2 fixation in the presence of 10^{-6} M IAA into the 80 % alcohol soluble components is much greater than that in the absence of IAA. IAA has apparently no effect on the incorporation of C^{14} into the 80 % ethanol insoluble components for the first 20 minutes.

The incorporation of C^{14} into such cell wall constituents as pectin, protopectin, polyuronide hemicelluloses, polysaccharides, and α-cellulose as affected by IAA is shown in table III. Though for the first 20 minutes the total incorporation of C^{14} into the alcohol insoluble fraction was unaffected by IAA, the relative distribution into the cell-wall constituents was altered. Thus, IAA stimulated C^{14}-incorporation into the polyuronide hemicelluloses and pectin, but had a retarding effect on C^{14} incorporation into some other constituents. When the incubation period was increased to 3 hours, a marked increase in the total C^{14}-activity of the alcohol insoluble fraction was observed, and the pattern of distribution found with the 20 minute experiments was altered to some extent. IAA continued to stimulate C^{14}-incorporation into the polyuronide hemicelluloses. C^{14}-fixation into pectin and protopectin was markedly inhibited by IAA, while that into polysaccharides and α-cellulose recorded a sharp rise. The metabolism of acetate-1-C^{14} and sucrose-U-C^{14} in relation to the effect of IAA on the synthesis of cell-wall components has been studied by Boroughs and Bonner (1) and Nance (7). From their observations, and on the basis of the data presented here concerning $C^{14}O_2$ fixation, it seems that IAA affects the utilization of different substrates for the synthesis of cell-wall constituents in a different matter and the level of the substrate may also be a determining factor (7). In the ether insoluble fraction of the 80 % ethanol extracts of Avena coleoptiles Yamaki (14) detected three groups of compounds in which C^{14} incorporation was stimulated by IAA. Unfortunately the chemical nature of these compounds is not known.

Examination of the alcohol soluble fraction of the 20 minute experiment revealed the presence of C^{14} in at least 17 compounds, some of which still remain to be identified. IAA stimulated the incorporation of C^{14} into most of the compounds; the stimulation was particularly remarkable in the case of citrate-isocitrate and malate. Among the compounds unidentified, mention should be made of a substance which gives certain color reactions characteristic of sugars and uronic acids. Its C^{14}-content was much less than that of many other compounds but its formation in such short periods of dark CO_2 fixation is of considerable interest. No radioactivity was detectable in gulonolactone, galacturonic acid, phosphogluconate, or free hexoses. With longer experimental periods, the pattern is very similar to that observed with 15 hours.

Naphthalene acetic acid (NAA) also stimulated CO_2 fixation as in the case of IAA. The pattern of C^{14} distribution, though similar in some respects, was, however, not identical. The stimulation was reflected largely in the citrate-isocitrate fraction and was not associated with a spectacular increase in malate or decrease in aspartate observed in IAA-treated coleoptile tissues.

CO_2 FIXATION BY AVENA ROOTS: The effect of IAA on CO_2 fixation by Avena root tissues, the growth of which is known to be inhibited by IAA, was also studied. One centimeter long sections of the roots of 4-day old seedlings were incubated with $NaHC^{14}O_3$ (20 μc/ml of experimental solution) for 7.75 hours in darkness, and extracted in 80 % ethanol as described in the case of coleoptile tissues. It was observed (table IV) that such low concentrations of IAA as 10^{-7} M brought about a six fold stimulation in the rate of $C^{14}O_2$ fixation. The distribution of C^{14} into individual compounds revealed many similarities with the pattern observed in coleoptile tissues. No decrease in C^{14}-activity in aspartate was, however, observed.

NATURE OF ACTION OF IAA ON CO_2 FIXATION: Largely on the basis of feeding experiments Yamaki

TABLE III

EFFECT OF IAA ON INCORPORATION OF C^{14} INTO CELL WALL COMPONENTS (% OF CONTROL; CONTROL = 100)

DURATION	TOTAL ALCOHOL INSOLUBLE	PECTIN	PROTOPECTIN	POLYURONIDE HEMICELLULOSE	α-CELLULOSE	POLYSACCHARIDE
20 min	98	272	48	139	101	42
3 hr	126	90	16	315	725	1,228
4 hr	222	50	5	333	1,542	525

TABLE IV

EFFECT OF IAA ON PRODUCTS OF $C^{14}O_2$ FIXATION IN AVENA ROOTS.*

COMPOUNDS	CONTROL	10^{-7} M IAA	10^{-5} M IAA	10^{-3} M IAA
Malate	2.2	17.6	20.4	19.7
Citrate, iso-citrate	4.0	51.3	75.5	16.4
Glutamate	0.9	10.6	8.2	4.2
Asparagine	3.2	6.9	6.6	7.3
Arginine	1.5	12.4	11.9	7.9
Methionine	1.3	7.4	3.7	2.7
Fumarate	1.4	11.1	14.4	3.6
Aspartate	0.7	3.3	4.1	2.7
Alanine	...	2.5	...	...
Total 80 % alcohol soluble	18.3	126.9	147.7	69.3

* Time 7 hr 45 min. (cpm/mg of tissue $\times 10^{-3}$)

(13) had concluded that, "the reaction of CO_2 consumption is not the same as the ordinary CO_2 fixation reaction, but instead, it might presumably be connected to some reaction that produces a substance different from those produced by the well known CO_2 fixation reaction". His findings were in agreement with the hypothesis that CO_2 fixation occurs during the formation of bound IAA, for the synthesis of which energy or supply of reduced pyridine nucleotides, is contributed by the respiratory reactions. These assumptions would result in an integration of CO_2 fixation, respiration, and the reactions involved in growth.

Whatever be the mechanism of CO_2 fixation in Avena coleoptiles, the auxin-induced stimulation of CO_2 fixation could be brought about by making available more of the acceptor of CO_2 fixation or some cofactor(s) required for the fixation process or increased metabolism of some of the products. When the coleoptiles were incubated with pyruvate-3-C^{14} or acetate-2-C^{14} in the presence of unlabelled $NaHCO_3$ (S. P. Sen, unpublished data) the C^{14}-distribution pattern was not identical with that observed in CO_2 fixation studies. Consequently greater attention was paid to cofactor studies. The most striking observation recorded in the present investigation was the remarkable accumulation of C^{14} in malate. If malate is formed through the agency of the TPN-linked malic dehydrogenase and IAA acts indirectly by stimulating metabolic reactions generating TPNH, then supply of TPNH should yield similar results. On the other hand if the DPN-linked malic dehydrogenase is the enzyme responsible, incorporation of DPNH in the experimental solution should give the desired results. This contention is further supported by the observation made in the present investigation that in the coleoptile tissues appearance of C^{14} in malate was associated with a marked disappearance of radioactivity in aspartate. Since IAA has been shown to be capable of coupling with coenzyme A (15) and this process may also be connected with the metabolism of bound IAA (12), it might also be of interest to study the effect of CoA on the auxin-stimulated CO_2 fixation process. Investigations were therefore carried out to study the effects of these compounds as also AMP and ATP (which are known to be concerned with the energy transfer mechanism of the cell) by incorporating them in the experimental solutions in the absence of IAA and comparing the effects to that observed with IAA.

The results are summarized in table V. Most of the co-factors stimulated CO_2 fixation, though in varying degrees; the distribution pattern of C^{14}, however, was not similar to what was observed with IAA. The two concentrations of IAA employed, namely 10^{-6} and 10^{-3} M, have widely divergent physiological effects; the former promotes growth and the latter inhibits it. In some cases the effects are reminiscent of the two phase concentration curves observed in growth, in other cases the co-factors are acting apparently as a nutriment, higher concentration stimulating the metabolic rates. Of the various co-factors tried, DPNH alone influenced the metabolism of CO_2 fixation products in a manner somewhat similar to that observed with IAA. The effect of IAA and DPNH on certain compounds concerned with the synthesis of malate is shown in table VI. The incorpo-

TABLE V

EFFECT OF DIFFERENT CO-FACTORS ON INCORPORATION OF C^{14} FROM $NaHC^{14}O_3$ IN 80 % ETHANOL SOLUBLE & INSOLUBLE FRACTIONS OF AVENA COLEOPTILES (% VARIATION FROM CONTROL)

TREATMENT	80 % ETHANOL SOLUBLE	80 % ETHANOL INSOLUBLE
Control	100	100
DPN, 8×10^{-7} M	125	148
DPN, 8×10^{-4} M	379	121
DPNH, 10^{-6} M	872	202
DPNH, 10^{-3} M	464	115
TPN, 10^{-6} M	118	62
TPN, 10^{-3} M	244	154
TPNH, 3.8×10^{-6} M	238	96
TPNH, 3.8×10^{-4} M	199	123
AMP, 10^{-6} M	159	96
AMP, 10^{-3} M	193	116
ATP, 10^{-6} M	150	132
ATP, 10^{-3} M	298	96
CoA, 2 mg/l	98	109
CoA, 200 mg/l	261	62
Thioctic acid 0.2 mg/l	116	62
Thioctic acid 200 mg/l	46	8
IAA, 10^{-6} M	1,547	126
IAA, 10^{-3} M	115	66

TABLE VI

EFFECT OF IAA & DPNH ON CERTAIN PRODUCTS CONCERNED WITH SYNTHESIS OF MALATE VIA CO_2 FIXATION IN AVENA COLEOPTILE (cpm/mg OF COLEOPTILE $\times 10^{-3}$

TREATMENT	MALIC ESTER	MALATE	ASPARAGINE	ASPARTATE
Control	Trace	Trace	Trace	9.9
10^{-6} M IAA	253.8	82.0	40.3	0.8
10^{-3} M IAA	56.2	25.2	8.7	0.2
10^{-6} M DPNH	70.9	31.5	18.3	1.0
10^{-3} M DPNH	29.7	17.1	11.6	0.4

ration of C^{14} into the cell wall components is also affected by IAA and DPNH in a similar manner (table VII).

Unfortunately, attempts to demonstrate the generation of DPNH in a cell-free system either directly, or by coupling the system to alcohol and lactic dehydrogenases, so far have not yielded reproducible results. In the cell free preparation, as also in the homogenates, the IAA stimulation of CO_2 fixation also could not be observed. Apparently during the process of homogenization certain factor(s) needed for the CO_2 fixation reactions and the IAA-induced DPNH generation process, (if such a process is operative in Avena coleoptile), was (were) largely destroyed. It is quite possible that the DPNH formed is almost instantaneously oxidized, and consequently no significant difference would be observed between the treated and control sets. In dealing with such feeding experiments involving the use of nucleotide type compounds it is important to know whether the nucleotides have entered at all. An appreciable decrease in DPNH concentration was observed at the end of the incubation period but no increase in the internal concentration could be demonstrated.

The mechanism of CO_2 fixation in Avena coleoptile has not been worked out as yet. In the present investigation it was found that in the absence of IAA the bulk of C^{14} incorporated was located in aspartate. Mazelis and Vennesland (3) have shown that phosphoenol pyruvate carboxylase and kinases are widely distributed in plant tissues. If it is assumed that CO_2 fixation in Avena coleoptiles also takes place through carboxylation of phosphoenolpyruvate to oxalacetate, and if the level of citrate-isocitrate and aspartate is taken as a measure of the oxalacetate formed, then it would appear that one of the major effects of IAA on CO_2 fixation is exercised through the reduction of oxalacetate to malate. This would involve a continuous supply of DPNH, and in fact use of DPNH as a substitute for IAA revealed a similar picture (table VI & VII). It would seem that IAA initiates a number of metabolic reactions which may lead to the generation of DPNH, a part of which is used for the synthesis of malate and for other reactions. The oxidation of DPNH could ultimately result in the formation of ATP which would then be utilized for a variety of energy consuming reactions. That one of the effects of IAA is to enhance ATP synthesis has recently been confirmed in this laboratory through the use of P^{32}-labelled phosphates (unpublished data).

TABLE VIII

EFFECT OF IAA ON FORMATION OF ASPARAGINE-C^{14} & GLUTAMINE-C^{14} FROM *l*-ASPARTIC ACID-G-C^{14} & GLUTAMIC ACID-l-C^{14}

	% AMINO ACID-C^{14} METABOLIZED	
	No IAA	IAA 10^{-6} M
Asparagine	0	54.5*
Glutamine	13.9	39.0**

* Coleoptiles first incubated with 10^{-6} M IAA in phosphate buffer of pH 6.0 for 75 min, then *l*-aspartic acid-G-C^{14} added (25 μc/ml). Incubation period with aspartic acid-G-C^{14} 3.5 hr.

** Coleoptiles incubated with 10^{-6} M IAA and glutamic acid-G-C^{14} (10 μc/ml) in phosphate buffer of pH 6.0 for 3 hr. Extraction with 80 % ethanol at 30° C.

The disappearance of aspartate in the presence of IAA in coleoptile tissues was, however, not exclusively due to the preferential formation of malate. In the presence of IAA, C^{14}-activity in asparagine was increased. It is possible that through an IAA stimulation of ATP synthesis, more of ATP was rendered available for amide synthesis. Experimental evidence in support of this contention is shown in table VIII.

TABLE VII

EFFECT OF IAA & DPNH ON INCORPORATION OF C^{14} FROM $NaHC^{14}O_3$ INTO 80 % ETHANOL INSOLUBLE CELL WALL COMPONENTS OF AVENA COLEOPTILE. TIME 4 hr (cpm/mg $\times 10^{-3}$)

TREATMENT	PECTATE	PROTOPECTIN	POLYURONIDE HEMICELLULOSES	α-CELLULOSE	POLYSACCHARIDES	RESIDUE LEFT AFTER OXALATE & 0.5 N HCl TREATMENT
Control	2.9	3.3	1.3	0.6	Trace	Trace
IAA 10^{-6} M	1.5	0.16	4.4	3.2	4.0	12.5
IAA 10^{-3} M	4.1	0.23	1.5	1.0	0.9	3.0
DPNH 10^{-6} M	1.1	0.04	3.0	0.9	3.7	6.1
DPNH 10^{-3} M	1.4	0.1	2.5	1.0	3.0	6.1

Note that incubation of the coleoptiles with aspartic acid-C^{14} or glutamic acid-1-C^{14} results in an increased incorporation of C^{14} into asparagine and glutamine, respectively. The lack of activity in glutamine in $C^{14}O_2$ fixation studies is probably due to the fact that any glutamine-C^{14} formed was destroyed during extraction with hot 80% ethanol, since glutamine is known to break down at 39° C.

From the data presented here one may conclude that auxin stimulates CO_2 fixation and influences the metabolism of the fixation products in a remarkable manner. The stimulation of fixation, however, does not appear to be correlated with the growth stimulus, since fixation is inhibited by lower concentrations of auxin than those which inhibit growth, and fixation is stimulated in root where auxin does not stimulate growth. It is also interesting that while a CO_2 concentration of 200 μg/ml would support auxin-induced stimulation of growth and CO_2 fixation by Avena coleoptiles, concentrations above 12.5 μg/ml are inhibitory to the binding of NAA-C^{14} to the proteins of pea internodes (11).

Summary

The relationship between CO_2 fixation and auxin-induced growth has been studied using $NaHC^{14}O_3$ as a source of $C^{14}O_2$.

Indole acetic acid in concentrations which are known to promote growth stimulates C^{14}-incorporation into Avena coleoptile tissues. The stimulation is maximal at a concentration of about 10^{-5} M; higher concentrations have an inhibitory effect. Naphthalene acetic acid also stimulates C^{14}-incorporation into coleoptile tissues.

In the presence of low concentrations of IAA the stimulation is detectable within a short period. IAA affects the contribution of CO_2 to the syntheses of cell-wall components in a varied manner: IAA stimulates the fixation into polyuronide hemicelluloses, polysaccharides, and α-cellulose with relatively long incubation periods; it has a distinct inhibitory effect on fixation into protopectin; the inhibition is marked after a few hours.

CO_2 is metabolized into a large number of compounds within a few minutes. After fixation for a few hours, the bulk of the C^{14} could be located in citric-isocitric acid and aspartic acid. In the presence of low concentrations of IAA the fixations into malic acid and asparagine were remarkably stimulated with an accompanying decrease in tagged aspartic acid. Such co-factors as DPN, TPN, DPNH, TPNH, AMP, ATP, and CoA, all stimulated the incorporation of C^{14}, but DPNH alone gave results which had some resemblance to the effects of IAA.

Indole acetic acid in concentrations which inhibit root growth also stimulates C^{14} incorporation by Avena roots in a manner largely similar to what is observed with coleoptile tissues.

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B. PRINTS FOR REPRODUCTION. After your manuscript has been accepted for publication you must submit good quality glossy prints (glossies) for making electrotypes.

1. Kinds of Illustrations. Figures are of two kinds, line drawings and halftones. Line drawings are just that—line drawings usually done with India ink on white paper. Halftones, in contrast to line drawings, are not just black and white, but they also are shaded from black to white. Because halftones are photographed through a screen some detail is lost in reproduction.

2. Special Treatment of Photographs. For illustrations in which it is especially important to bring out all the detail present in the original photograph special processing will be undertaken. Should your paper contain electron-micrographs, radioautographs, or other material whose reproduction should be deemed critical, call the editor's attention to the need for special processing.

3. Good Illustrations. Plan in advance for clear, legible illustrations. Line drawings should be intensely black on white; halftones should have good contrast and be in sharp focus throughout. No glossy should contain blemishes. Do not use paper clips on photos: grooves, gouges, and creases show when reproduced.

Generally the appearance of a graph is improved if the top and right sides are boxed in by lines of the same weight as the abscissa and ordinate.

Guided lettering is preferable to hand lettering or typing.

4. Size. Although the Editor determines the final printed size of an illustration, an author should plan for maximum reduction. If symbols, such as triangles, circles, and squares (or letters & numerals) are not sufficiently large to begin with, they may become mere blurs when reproduced. Any letter smaller than 1 mm may be illegible. Exceedingly large symbols, thick lines, or heavy numerals may become unsightly when reduced.

Figures are reproduced the same size (x 1) or they are reduced. (Enlarging magnifies defects.) Illustrations are fitted either into 3 inch or 6⅛ inch column widths. Although

occasionally an illustration must be 8½ inches high, this is undesirable. One should allow space at the bottom for the figure legend. It may help you to sketch a rough onion skin overlay of a typical Journal page layout for estimating final reduced size of an illustration. Final size, however, cannot be estimated until after illustrations are properly grouped and mounted.

5. Grouping. One large illustration is cheaper to reproduce than three to six small ones, so *as far as possible group all figures.* Mount similar figures close together. Place halftone and line drawings in separate groups.

Limit the area of each photograph to that which is necessary to show pertinent details. Trim off irrelevant portions and minimize blank space.

6. Mounting. The placement of figures is the author's responsibility; the engraver does not alter the arrangement. Mount all illustrations with rubber cement on white cardboard or stiff white paper. Total size should not exceed 9 x 11 inches. Anything larger than an ordinary file folder is easily mutilated; it also requires special packaging and handling.

7. Identification. Place the author's name on the back of each figure and legend. Place the figure number on the back of each figure. Identification insures against misplacement.

V. SUBMITTING MANUSCRIPTS.

Send the original copy of the manuscript, the first carbon, and two copies of all illustrative material to the Editor, Dr. Allan H. Brown; Department of Botany; University of Minnesota; Minneapolis 14, Minn. Retain a carbon copy of your manuscript and original photographs or drawings to insure against loss.

PLANT PHYSIOLOGY does not accept a manuscript which has not been submitted directly by an author. If your manuscript is submitted by someone other than one of the authors, send a letter to this office stating that you wish to publish that particular manuscript in PLANT PHYSIOLOGY. Your manuscript cannot be processed until this letter is received. This precaution protects you as well as the Journal.

VI. PROCESSING MANUSCRIPT.

A. REVIEW. The manuscript usually will be sent to two reviewers familiar with that field of research covered by the paper. Each reviewer evaluates the manuscript, suggests improvements, and recommends accepting or rejecting the paper. Usually two months suffice for review and decision. When there is marked disagreement between reviewers, the Editor may appoint a referee (often a member of the Editorial Board) to evaluate the manuscript in light of the reviews; the referee recommends either for or against acceptance.

B. REVISION. Manuscripts returned to the author for revision should be sent back to the Editor within two months; otherwise the paper will be published under the date of receipt of the revised manuscript and not the original date.

Retype each page on which there are extensive changes.

C. GALLEY PROOF. This will be sent to the person designated on page 1 above the title. (See II D 1).

1. Returning Proofs. Return only one set of galley proofs to Editor. Keep any other set for reference. Be certain to notify Editor promptly of change of address, so galleys can be sent to the correct address.

2. Charges for Errors. You will be charged at cost for all changes or corrections in galley and page proof which are your fault. You will not be billed for errors which are not your fault. Here are two ways to minimize charges:

a. Proofread Manuscript. Check ALL references carefully BEFORE submitting manuscript to Editor. Corrections in typing are easy to make, whereas corrections in galley or page proof are expensive and sometimes difficult to make.

b. Corrections. PLANT PHYSIOLOGY charges 45c for correcting a straight line of copy; 90c for a line of literature citation or table, $1.80 for a line of formula. Where a correction is optional remember that a change made near the beginning of a paragraph can necessitate resetting every subsequent line to the end of the paragraph. You will be billed for each line the printer must reset to correct an error (this may be a dozen lines), so weigh your cost against the importance of each change you propose.

D. DELAYS. In general, a manuscript should be reviewed and returned to you with the Editor's decision within two months. After you send a revised manuscript to the Editor allow 2½ months for galleys to be sent you for correcting. Should you find that processing your manuscript has taken an unusually long time at either stage, it would be well to inquire of the Editor as to the cause of the delay. (This is a precaution against unrecognized loss of manuscripts in the mail—a rare event, but it happens.)

E. ERRATA. If necessary, an errata will be published for each volume. Authors therefore are requested to call the attention of the Editor to any significant errors in their published manuscripts.